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Regulation Of Immune Responses To Self And Nonself

Angela Panoskaltsis

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REGULATION OF IMMUNE RESPONSES TO SELF AND NON-SELF

by

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ABSTRACT

B cells are regulated early upon exposure to antigen by negative Fc signals generated by the crosslinking of antigen receptors with Fc receptors which is mediated by antigen-IgG-antibody complexes. This negative end-product feedback can be overcome by T helper cells, but in their absence, agents which bind to the Fc portion of IgG can replace the need for T cell help. Rheumatoid factor (RF) is a naturally occurring antibody with specificity for IgG-Fc and was investigated for its ability to block negative Fc signals. Monoclonal murine RF was shown to reconstitute, in an antigen-specific and dose-dependent fashion, both the primary and secondary T-dependent antibody response of T cell-depleted murine spleen cell cultures. In order to see this effect, cultures had to be antigen stimulated and T cell depleted. Reconstitution by RF could be abrogated by the addition of non-specific, intact, murine IgG and not by $F(ab')_2$ or IgM.

The dysregulation of autoimmune responses was also investigated. It was demonstrated that normal, nonautoimmune-prone mice generated spontaneous anti-ssDNA antibodies *in vitro* of higher avidity than autoimmune-prone mice as determined by plaque inhibition assay using free ssDNA. Addition of RF to spleen cell cultures of normal mice resulted in a decrease of the IgM-anti-ssDNA antibody avidity as measured by both plaque inhibition and competitive inhibition ELISA assays. These results and the presence, *in vivo*, of low levels of autoantibody from high avidity antibody-producing cells in normals and high levels from low avidity antibody-producing cells in autoimmune-prone mice indicate that negative Fc signalling plays a role in the resulting spectrum of antibody avidities and possibly of class by being a key control element in the IgM to IgG switch.

This thesis offers a novel role for RF as an Fc signal blocking agent, but more importantly, implicates end-product feedback by endogenously produced IgG as a key immunoregulatory mechanism during critical stages of B cell activation.

Furthermore, inefficient negative Fc signal transmission may be the prime deregulating element leading to conditions conducive for the emergence of clinical autoimmunity.

DEDICATION

To my mother

Christina Lytrides

(nee Spyropoulou)

who raised me with

two strong hands

and

a soft heart

and in memory

of my father

Nicholas Panoskaltsis

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LIST OF ABBREVIATIONS

α	Anti
Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
B cell	Bone marrow-derived lymphocyte
BSA	Bovine serum albumin
BSS	Balanced salt solution
B/W	NZBxNZW F1 (first generation progeny)
CH_3	Constant heavy 3 domai
CMEM	Complete minimal esse ial medium
Con A	Concanavalin A
DNA	Deoxyribonucleic acid
DG	Diacylglycerol
dsDNA	Double-stranded DNA
Fab, $F(ab')_2$	Antibody-binding fragment
FACS	Fluorescence activated cell sorter
Fc	Crystallizable fragment
$Fc\gamma R$	Receptor for Fc of gamma heavy chains of IgG
$Fc\mu R$	Receptor for Fc of mu heavy chains of IgM
FCS	Fetal calf serum
F/H	Ficoll-hypaque
IBF	Immunoglobulin-binding factor
IP_3	Inositol triphosphate
Ig	Immunoglobulin
sIg	Surface immunoglobulin
IL-4	Interleukin 4
LPS	Lipopolysaccharide
2-ME	2-Mercaptoethanol
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
NZB	New Zealand black mice
NZW	New Zealand white mice
OXRBC	Ox red blood cells
PBS	Phosphate-buffered saline
PFC	Plaque forming cells
PKC	Protein kinase C
RF	Rheumatoid factor
SLE	Systemic lupus erythematosus
SRBC	Sheep red blood cells
ssDNA	Single-stranded DNA
TRF	T cell-replacing factor-containing supernatant

CHAPTER 1 HISTORICAL REVIEW AND INTRODUCTION

1.1 INTRODUCTION

The immune system can best be described as a diffuse but organized structure in which the well-orchestrated interactions among its components elicits appropriate responses for the immunocompetent host. It is not difficult to lose the focus of this definition, especially when looking through immunology journals, but so long as one keeps in mind that the system is made up of regulators and effectors and that each is also a "regulatee", it soon becomes clear (or less foggy) that most of the immune system is actually redundant. Many cells and mediators or factors are pleiotropic and the effects of physiochemically different molecules on one cell can result in the same outcome, a case of "the end justifies the means". The immune system has evolved to entail the use of many "backup" systems to ensure the proper outcome ensues after antigenic stimulation. This means, of course, that the immune system is centered around a minimal number of basic control mechanisms which are crucial to the regulation of the immune response. The role of one of these minimal, often overlooked, regulatory mechanisms in the response to foreign and autoantigens is the epicenter of the present study.

This thesis concerns the regulation of the humoral or antibody-mediated immune response as opposed to the cell-mediated arc which can involve antibody (hence, the two can be connected) but as a subordinate player. Via binding by their variable Fab regions, antibodies are among the few types of molecules that recognize antigen and, hence, can serve to focus antigen to a suitable antigenic environment and also can carry out various effector functions via the antibody "tail" or Fc portion. The antibody-producing B cells, although amenable to regulation by other cells such as antigen-presenting cells and T cells either by direct cell contact or via mediator molecules (factors or lymphokines), can also be regulated by their end-product, that is, antibody, in a feedback mechanism that is not only simple but

antigen-directed. Membrane-bound antibody serves as the antigen receptor for the B cell which also expresses receptors for the other effector component of the antibody molecule, the Fc portion, and is therefore called the Fc receptor. The crosslinking of these receptors leads to inactivation of the B cell and the effect of preventing the IgG-Fc/Fc receptor interaction from occurring is the subject of this thesis.

1.2 B CELL ACTIVATION

In their response to antigen, B cells follow a course of proliferation and differentiation which, for T-dependent antigens, requires T cell help, and minimally so for T-independent antigens. Four main models of B cell activation and the role of surface immunoglobulins (sIgs) have been proposed.

Coutinho and Moller (1974) proposed a one-signal model in which the main role of sIg was to bring antigen to the cell surface. A T-independent antigen (which is usually mitogenic for B cells) could then deliver the proliferative and differentiative signal, while T dependent antigens could also focus T cells to the B cell surface and, hence, allow cognate help to occur.

The "critical matrix" model, proposed by Diener and Feldmann (1972), is also a one-signal model in which antigen is presented in a multivalent form by macrophages. If T-dependent, the antigen is bound to the macrophage by T cell factors which recognize carrier determinants. The multivalent antigen then crosslinks the surface Igs on the B cell and, in doing so, triggers the B cell. The Coutinho and Moller model did not require this crosslinking event.

A two-signal model was proposed by Bretscher and Cohn (1970) in which the first signal was provided by the crosslinking of sIg by antigen and delivered a negative stimulus (Signal 1). Proliferation and differentiation was induced by Signal 2 which was provided by T cells or their factors and reversed the negative Signal 1. B cell unresponsiveness would result if the second signal was not received.

Subsequently, this model was further refined to suggest that both Signals 1 and 2 occurred during B cell activation before proliferation and maturation signals (Cohn, 1985). Attachment of antigen to the antigen receptor delivers a negative signal to the B cell (Signal 1) and associative recognition of the antigen by a helper T cell results in the delivery of Signal 2 -- a derepressive signal -- and, hence, activation. Proliferation and maturation are then induced by lymphokines in the presence of antigen. Thus, self-nonself discrimination occurs outside of primary lymphoid organs and is governed by the T helper/antigen-reactive cell interaction. If Signal 2 is not delivered, inactivation results.

A fourth model was recently published (Sinclair and Panoskaltsis, 1987a) where initial crosslinking of antigen receptors is an activation signal, and the negative signal, described by Bretscher and Cohn as Signal 1 and mediated through the Ag receptor, is mediated by endproduct antibody and is Fc-mediated. The negative signal is mediated through the Fc receptor as long as crosslinking of Ag- and Fc-receptors has occurred. Therefore, the positive signal mediated by the Ag receptor was called Signal < 1, and the inhibitory Fc-mediated signal was termed Signal 1 in keeping with the negative Signal 1 of Bretscher and Cohn. Thus, Signal 1 follows Signal < 1 and will inactivate the B cell if T cell help is not received. Hence, Signal 2 is a derepressive signal and mediated by T cell help providing the antigen is presented in a manner conducive to B-T cell collaboration. This puts the onus, on whether an immune response will be mounted, at the level of proper antigen distribution and presentation. That is, B cells normally respond to self as well as to non-self antigens but anti-self B cells remain inactivated because self is either presented in a manner which prevents effective B-T cell interaction or end product feedback is at such a high level that it is irreversible. As an alternative to T cell help, agents which block the Fc-FcR interaction can prevent negative Fc signals and curb the need for Signal 2.

Studies on B cell activation by surface Ig crosslinking demonstrated that the binding of antigen receptor to its ligand stimulates the phosphatidylinositol hydrolysis cycle (Coggeshall and Cambier, 1984) by virtue of being coupled to a guanine nucleotide-binding (G) protein (Klaus *et al.*, 1987). This hydrolysis liberates inositol triphosphate (IP_3) and diacylglycerol (DG) which are second messengers that play major roles in various cellular processes such as secretion, metabolism and cell growth (Berridge and Irvine, 1984). Diacylglycerol activates protein kinase C (PKC) which is thought to mediate the translation of signals received by the cell into intracellular responses (Nishizuka, 1984). Inositol triphosphate, on the other hand, mobilizes calcium from intracellular stores (reviewed by Berridge and Irvine, 1984). The increase in free intracellular calcium increases MHC Class II expression (Ransom and Cambier, 1986) which, in turn, enhances the interaction of B cells with Class II-restricted, antigen-specific helper T cells whose signals induce B cells to complete the cell cycle and engage in full-scale antibody production (Janeway *et al.*, 1984). The crosslinking of antigen receptors with Fc receptors uncouples G proteins from the antigen receptors (Rigley *et al.*, 1989), inhibits the breakdown of inositol phospholipids (Bijsterbosch and Klaus, 1985), calcium mobilization, the induction of DNA synthesis and the increase in c-*myc* expression which usually accompanies progression into the G_1 phase of the cell cycle (Phillips and Parker, 1987). It is noteworthy that lipopolysaccharide (LPS), a polyclonal B cell activator, does not induce the breakdown of inositol phospholipids (Bijsterbosch *et al.*, 1985) and the second messengers induced by LPS are not known although it may directly activate PKC (Wightman and Raetz, 1984; Chen *et al.*, 1986). Thus, more than one biochemical pathway is available for B cell activation and studies on B cell regulation using polyclonal activators such as LPS cannot possibly elucidate the control mechanisms operating in a normal, antigen-specific, sIg-mediated, immune response.

1.3 REVIEW OF ANTIBODY-MEDIATED IMMUNOSUPPRESSION

It is accepted that B cells are very sensitive to inhibition by low doses of passively administered IgG antibody specific for the immunizing antigen. This suppression was first thought to be due to antigen masking so that antigen-sensitive cells could not be stimulated (Uhr and Moller, 1968). However, it was demonstrated (Sinclair *et al.*, 1968; Sinclair, 1969; Gordon and Murgitz, 1975) that intact IgG was more effective at immunosuppressing that the $F(ab')_2$ fragment of antibody. In fact, $F(ab')_2$ fragments could interfere with suppression mediated by the intact antibody (Chan and Sinclair, 1973) which indicated that the poor inhibition by $F(ab')_2$ was not due to its inability to bind to antigen. Even as an antigen masking agent, $F(ab')_2$ antibody was not a good immunosuppressant (Sinclair *et al.*, 1970), as multiple injections of $F(ab')_2$ could not terminate an immune response. In contrast, only one injection of intact IgG was effective, thus ruling out rapid excretion of $F(ab')_2$ antibody as a reason for its ineffectiveness as an immunosuppressant. In fact, only one injection of $F(ab')_2$ was required to competitively interfere with immunosuppression by intact antibody (Chan and Sinclair, 1973). These experiments demonstrated the importance of the Fc portion of antibody in antibody-mediated suppression after the binding of antibody to antigen.

Many forms of antibody or immunoglobulin-mediated immunosuppression are Fc-dependent. These include suppression by 1) antibody specific for antigen, hapten or carrier (Henney and Ishizaka, 1970; Kappler *et al.*, 1971; Sinclair *et al.*, 1974; Hoffmann and Kappler, 1978); 2) anti-antigen receptor antibody (Sidman and Unanue, 1976; Phillips and Parker, 1983); 3) anti-isotype (reviewed in Cooper *et al.*, 1980); 4) anti-allotype (Cinader and Dubiski, 1976); 5) anti-idiotype (Pawlak *et al.*, 1973; Kohler, 1980); 6) haptenated immunoglobulin (Waldschmidt *et al.*, 1983); 7) insolubilized Ig complexes (Ryan and Henkart, 1976) and 8) aggregated Ig

complexes (La Via and La Via, 1978). Prevention of Rh disease is also mediated by an Fc-dependent mechanism through the administration of IgG-anti-Rh antibodies to the mother (Chown, 1969).

Studies on anti-allotypic antibodies showed that suppression was dependent upon the CH_3 domain of the Fc portion of IgG (Connell and Dubiski, 1977). The CH_3 domain is responsible for cytophilic activity on B cells and is not involved in complement activation and, therefore, the complement pathway cannot be considered to play an exclusive role in antibody-mediated suppression. Furthermore, IgG-mediated suppression was found not to correlate with complement-activating ability so that elimination of antigen by complement-dependent phagocytosis was ruled out as the effector mechanism (Heyman *et al.*, 1988; Wiersma *et al.*, 1989). Other studies demonstrated that removal of cytophilic activity (by adsorption) also removed the immunosuppressive activity (Ivanyi, 1970).

There have been conflicting reports as to which IgG subclasses augment or suppress immune responses in studies on anti-idiotypic antibodies (Eichmann, 1974; Rajewsky and Eichmann, 1977), antibodies specific for antigen (Gordon and Murgita, 1975) and on the effects of antibody subclass on tumour or allograft survival (Voisin, 1980). IgM antibody does not immunosuppress in an Fc-dependent fashion (Henry and Jerne, 1968; Dennert, 1971).

It has now been resolved that, in fact, all IgG subclasses are effective in Fc-mediated suppression which is correlated with antibody affinity (Heyman and Wigzell, 1984; Wiersma *et al.*, 1989) and presence of carbohydrate chains on the Fc portion (Heyman *et al.*, 1985). It has been demonstrated that the Fc receptor binding sites on IgG are highly conformation-dependent and, since IgG-Fc conformation was not affected by glycosylation, it has been suggested that the carbohydrate moieties simply stabilize the structure of the Fc portion and do not

interact with Fc receptors (Walker *et al.*, 1989). However, the requirement for carbohydrate for the delivery of negative Fc signals argues against this suggestion.

Recently, Fc-dependent enhancement of the immune response was demonstrated but this augmentation was correlated to isotype and complement activation (hence, better antigen presentation due to binding to C3b receptor) and not to affinity for antigen (Wiersma *et al.*, 1989).

Having determined that immune responses could be inhibited by antibody via an Fc-dependent mechanism, Sinclair and Chan (1971) developed the "tripartite inactivation model". This model proposed that upon binding antigen (attached to the antigen receptor), inhibitory antibody also delivers negative signals via the binding of its Fc portion to an Fc receptor on the B cell. That is, the crosslinking of antigen receptors and Fc receptors inactivates B cells. Since this model was first put forth, Fc receptors have been discovered on B cells (Paraskevas *et al.*, 1972; Dickler and Kunkel, 1972; Basten *et al.*, 1972) and it has been determined that "tripartite" complexes can directly inactivate B cells (Sinclair *et al.*, 1974; Oberbarnscheidt and Kolsch, 1978; Stockinger and Lemmel, 1978).

Numerous investigators have since confirmed these findings and have demonstrated suppression via Fc-dependent crosslinking of Ag receptors and Fc receptors in various systems - by antigen-antibody complexes (Klaus and Abbas, 1978; Kolsch *et al.*, 1980; Voisin, 1980) and by intact antibodies of a suppressive isotype bound to surface Ig (Sidman and Unanue, 1976; Braun and Unanue, 1980; Tony and Schimpl, 1980; Philips and Parker, 1983). The latter groups also demonstrated that concomitant occupancy of antigen receptors and Fc receptors, on its own, did not induce suppression; that is, crosslinking was mandatory. Recently, Uher and Dickler (1986a) showed that independent but simultaneous crosslinking of each of the two receptors by separate ligands inhibited differentiation while crosslinking by one ligand which could itself bind to both the antigen receptor and

Fc receptor inhibited proliferation and differentiation. Dominance of the negative Fc signal was demonstrated by Tony and Schimpl (1980) when they showed that $F(ab')_2$ fragments of anti-sIg antibody (which were stimulatory) could be made suppressive upon binding intact antibody to them. That is, the $F(ab')_2$ fragments now contained an Fc. Sidman and Unanue (1976) also proved that the negative signal was a trans-membrane one since capping and endocytosis of the inhibitory complexes were not necessary to deliver the signal.

It should be noted that not all forms of Fc-dependent suppression need operate by the same mechanism. For example, the suppression observed using insolubilized Ig complexes (Ryan and Henkart, 1976) could have been due to destruction of the B cell surface membrane upon removal of B cells from the complexes. Opsonic mechanisms have also been implicated in other forms of antibody-mediated suppression. This mechanism entails the binding of antigen-antibody complexes onto an antigen-reactive cell via the antigen receptor. The Fc of the antibody in the complex is then bound by macrophage Fc receptors and the antigen-reactive cell is engulfed and destroyed by the macrophage (reviewed in Hutchinson, 1980). However, only T cells, not B cells, have been shown to be involved in this form of immunosuppression (Hutchinson *et al.*, 1983). Furthermore, Fc-dependent suppression can occur independently of macrophages or T cells so that the involvement of these cells can be ruled out (Abbas *et al.*, 1977; Masuda *et al.*, 1978; Oberbarnscheidt and Kolsch, 1978; Kolsch *et al.*, 1980; Kim *et al.*, 1983; Phillips and Parker, 1983; 1985).

Recent experiments have shown that prevention of the crosslinking of antigen receptor and Fc receptor allows full activation of B cells to be attained. This has been done with monoclonal antibodies against the Fc receptor (Lamers *et al.*, 1982; Phillips and Parker, 1984) and with staphylococcal protein A (SPA) which binds to the Fc portion of rabbit IgG (Tony and Schimpl, 1980; Phillips and Parker,

1983; Bijsterbosch and Klaus, 1985). Lamers *et al.* (1982) were able to induce full B cell activation with their anti-Fc receptor antibody (2.4G2) in the absence of T cells. However, this preparation was subsequently shown to have been contaminated with a mitogen (Lamers *et al.*, 1984). Even when further purified, this antibody not only induced proliferation (as mitogens do) but also antibody production. Since these experiments were done using T cell depleted murine spleen cells, it is possible that the main trigger for antibody production is the prevention of negative Fc signal transmission and that this could be mediated by T cells.

Adoptive transfer experiments involving the transfer of bone marrow cells and antigen demonstrated that antibody-mediated suppression could be inhibited by thymus cells (Sinclair *et al.*, 1976). Allogeneically activated T cells have been shown to interfere with Fc-dependent suppression (Lees and Sinclair, 1975) and Hoffmann and Kappler (1978) demonstrated that the presence of T cell factors (AEF - allogeneic effect factor, or TRF - T cell replacing factor) alone can prevent inactivation mediated by the Fc of antibody. TRF does not bind to Fc receptors (Takatsu *et al.*, 1982; Tominaga *et al.*, 1982) and, therefore, does not sterically block the interaction between the Fc receptor and the Fc portion of IgG as was previously thought (Schimpl *et al.*, 1977).

1.4 Fc-MEDIATED REGULATION BY ENDOGENOUSLY PRODUCED IgG ANTIBODY CAUSES NEGATIVE FEEDBACK

The experiments mentioned above were, and erroneously still are, referred to as "negative feedback" studies but, since they involved passively administered suppressive antibody, they only showed that IgG antibody had potential as an immunosuppressive agent. The model became more significant as an immunoregulatory mechanism when Fc-mediated regulation of antibody production was demonstrated to occur via *endogenously* produced IgG antibody (Sinclair, 1983a; Sinclair and Panoskaltsis, 1986a).

It is well known that, in a primary immune response, B cells initially produce IgM and then IgG and that T cells are required for the IgM to IgG switch. Adoptive transfer experiments (Chan and Sinclair, 1971) in which bone marrow cells and a T dependent antigen (sheep red blood cells - SRBC) were injected into lethally irradiated syngeneic mice have shown that the addition of high concentrations of T cells shifted the response from IgM to IgG synthesis. In low T cell concentrations, the shift did not occur; that is, the IgM response prevailed. However, the presence of intermediate T cell concentrations led to no response at all. This suggested that the IgM to IgG switch did occur but that something was suppressing the response. This led to the hypothesis that the small amounts of IgG initially produced could suppress the B cells by the binding of IgG-Fc to the Fc receptor and that not enough T cells were present to interfere with this inhibition.

Therefore, the distinction must be made between negative feedback which refers to suppression mediated by *endogenously* produced end-product antibody (specifically, IgG) and antibody-mediated immunosuppression which is caused by passively administered antibody.

Preliminary evidence that antibody produced early in an ongoing immune response can suppress a later response was found when antibody from the time of the first peak response suppressed the appearance of the second peak (Britton and Moller, 1968). Although these responses involved primary IgM production, one cannot rule out that a small amount of suppressive IgG was present in the first peak. These investigators also found that IgG was more potent at this suppression than IgM and they suggested that the cyclical variations of antibody production observed could be explained by an antibody feedback mechanism which regulated the primary immune response.

IgG antibody is produced in response to T cell dependent antigens in the presence of sufficient T cell help (Isakovic *et al.*, 1965; Sinclair, 1967), whereas IgM

production can be T cell independent (Mitchell *et al.*, 1972). However, T cell independent IgG responses have been induced in athymic nude mice (Mosier *et al.*, 1974; Rude *et al.*, 1976; Humbert *et al.*, 1979), that is, in the relative absence of T cells, so that the possibility of IgG production occurring early during a T-dependent IgM response cannot be excluded. In fact, those IgM responses which are T dependent may be so because T cell help is needed to interfere with feedback mediated by small amounts of immunosuppressive IgG produced early in the immune response (Sinclair, 1983a).

In order to determine whether end-product negative feedback by antibody could be demonstrated early in an ongoing immune response, it was assumed that the feedback was mediated by IgG antibody, that it was Fc-dependent and interfered with by helper T cells or their factors. Directly measuring the very tiny amounts of IgG produced early during a primary *in vitro* response, although difficult, was pointless since one could not determine whether that antibody measured was the one which would have inhibited the immune response by negative feedback. However, since it was likely that end-product feedback was Fc-dependent, then blockade of Fc signals should prevent the feedback. This was an indirect, but more definitive, method of determining the presence of immunoregulatory IgG. Because T cell help interferes with Fc-dependent negative feedback, it was postulated that the main, or rate-limiting, role of helper T cells may be to prevent negative Fc signalling or end-product feedback. It was shown that blocking Fc signals with heterologous goat-anti-mouse IgG-Fc antibodies allowed T cell *depleted* mouse spleen cells to mount a T *dependent* primary antibody response (Sinclair and Panoskaltsis, 1987c). Prevention of the IgG-Fc:Fc receptor interaction may have also been accomplished by use of an anti-Fc receptor antibody, but since this antibody binds directly to the B cell surface, the distinction could not have been made whether it was preventing negative Fc signals from occurring or positively

triggering the B cell via another pathway. In fact, those goat-anti-Fc antibodies which were most effective at reconstituting the T dependent anti-SRBC response of T depleted cultures were those which could not bind to the B cell surface as demonstrated by fluorescence activated cell sorter (FACS) analysis and, hence, had exclusive specificity for determinants exposed on the Fc portion of *secreted* IgG and not on surface immunoglobulin (Sinclair and Panoskaltsis, 1989b). The effect of Fc signal blockade could only be seen in cultures which were T cell poor and antigen stimulated. T cell depletion was necessary because, otherwise, T cell-replacing factors would interfere with Fc signalling and the presence of Fc-blocking agents would be of no consequence. Antigen stimulation was needed in order to obtain an antigen-specific response and to generate the Ag-Ab complexes required of the "tripartite" inactivation model. In order to reconstitute, the goat-anti-mouse-Fc antibody had to be present from culture initiation up to 72 hours (3 days). Furthermore, increasing amounts of anti-Fc antibody were needed from days 0 to 3 (100 picograms or 10^{-12} M at day 0 up to 300 nanograms or 3 X 10^{-9} M on day 2).

1.5 SCOPE OF THESIS

The obvious biological counterpart of the anti-Fc antibodies used in previous studies is rheumatoid factor (RF). RF is an autoantibody, usually of the non-immunosuppressive IgM isotype, specific for the Fc portion of IgG (Nemazee and Sato, 1982; 1983; Van Snick and Coulie, 1983; Coulie and Van Snick, 1983; 1985; Theofilopoulos *et al.*, 1983; Nardella *et al.*, 1985; Nemazee, 1985). It has been found that RF has higher affinity for IgG in immune complexes than for free IgG (Van Snick and Coulie, 1983) and, thus, recognizes an Fc determinant which is induced or exposed after binding to antigen (Nemazee and Sato, 1982). RF is specifically induced by immune complexes interacting with the RF-producing cell (Nemazee and Sato, 1983; Coulie and Van Snick, 1983; 1985).

Many facts point to the possibility that RF can be an immunoregulatory molecule. In normal, as well as autoimmune, subjects, a large proportion of B cells produce antibody specific for IgG antibody attached to antigen (Nemazee and Sato, 1982; Van Snick and Coulie, 1983; Hobbs *et al.*, 1985). Also, during secondary immune responses, the amount of RF produced was found to equal or exceed the amount of IgM antibodies specific for the immunizing antigen (Coulie and Van Snick, 1983; Van Snick and Coulie, 1983), but its production peaked and subsided earlier (Coulie and Van Snick, 1983; Nemazee, 1985).

As an alternative to T cell help, agents which can block Fc signals, such as RF, may not only allow T cell dependent responses to foreign antigens, but also to autoantigens, to be mounted. It is therefore predicted that murine rheumatoid factor (IgM-anti-IgG-Fc antibody) will reconstitute the T-dependent responses of T cell depleted cultures and, hence, replace T cells in the same manner as the heterologous goat-anti-mouse-Fc antibodies studied previously. If demonstrated, this would add a new understanding to B cell regulation since all the elements used in this demonstration are natural components of the immune response, including RF as an Fc signal blocking agent. The emergence of autoimmune diseases may in fact be due to aberrations of this Fc signalling mechanism. Furthermore, clues to the control of these disease states may be found within the framework of the negative Fc-signalling apparatus.

CHAPTER 2 IMMUNOREGULATION BY RHEUMATOID FACTOR - AN Fc SIGNAL BLOCKING AGENT

2.1 INTRODUCTION

It has been demonstrated that heterologous antibodies produced against the Fc portion of murine IgG have the capacity to reconstitute a T-dependent response of murine spleen cell cultures which have been partially depleted of their T cell population (Sinclair and Panoskaltsis, 1986a; 1986b; 1987a; 1987c). The rationale for these investigations was based on the following: (a) the Fc portion of antibody is required for immunosuppression (Sinclair, 1969; Lees and Sinclair, 1973; Wason and Fitch, 1973), (b) T cells (Lees and Sinclair, 1975; Sinclair *et al.*, 1976) and their factors (Hoffman and Kappler, 1978; O'Garra *et al.*, 1987) interfere with Fc-dependent immunosuppression, and (c) antibody, produced endogenously in an immune response, may be an important feedback regulator (Sinclair, 1983a). These earlier studies (Sinclair and Panoskaltsis, 1986a; 1986b; 1987a; 1987c) were prompted by prior observations and hypotheses, and confirmed a previously published prediction (Sinclair, 1983a). The results were interpreted to indicate that prevention of Fc-mediated antibody feedback is a rate-limiting step in T-B cell collaboration.

A goat anti-mouse IgG-Fc antibody is not a normal component in murine responses. However, murine rheumatoid factors (anti-IgG-Fc antibodies) are regularly produced both in normal responses and in various autoimmune states (Van Snick and Coulie, 1983; Coulie and Van Snick, 1985; Nemazee and Sato, 1982; 1983; Nemazee, 1985; Monestier *et al.*, 1986; Theofilopoulos *et al.*, 1983; Stanley *et al.*, 1987). Therefore, the ability of murine RF, directed against the Fc portion of IgG, to reconstitute T-depleted cultures in their responses to T-dependent antigens, is the subject under investigation in this chapter. The results demonstrate that this

naturally occurring Fc-binding antibody within the murine lymphoid system is capable of overcoming deficiencies in T cell numbers.

2.2 MATERIALS AND METHODS

2.2.1 Mice

CBA/J mice were purchased from Jackson Laboratories, Bar Harbor, Maine, and DBA/2J from Charles River Canada Inc., St. Constant, Quebec. Mice were maintained on an oral diet of water and cubed food. At times of short supply, CBA/J mice were kindly provided by Dr. G. H. Strejan. CBA/J mice were between 6-8 weeks and DBA/2J mice 6-16 weeks of age when used.

2.2.2 Media for Spleen Cell Cultures

All media and supplements were obtained from Gibco, Burlington, Ontario unless otherwise specified and were sterilized by filtration using a 0.22 um filter (Millipore Corporation, Bedford, MA).

Complete minimal essential medium (CMEM pH 7.2) consisted of (v/v) 84% MEM (9.4 g/l distilled water, pH 4.2), 10% fetal calf serum (FCS), 1% sodium pyruvate (100 mM), 1% non-essential amino acids (10 mM), 1% L-glutamine (200 mM), 0.5% gentamycin (10mg/ml) and 3% sodium bicarbonate (7.5% solution -- J. T. Baker Chemical Co., Phillipsburg, NJ). A medium with reduced supplement and FCS requirements was also used -- 96% Opti-MEM (13.6 g/l dH_2O, 2.4g $NaHCO_3$/l, pH 7.0), 2% FCS, 1% non-essential amino acids (10 mM) and 0.5% gentamycin with 2-mercaptoethanol (2-ME -- Sigma, St. Louis, MO) at a concentration of $5X10^{-5}M$. Use of complete Opti-MEM did not in any way affect either cell viability or antibody responses as compared to CMEM.

Hanks' balanced salt solution (BSS) was made by dissolving 9.8g BSS powder and 0.35g $CaCl_2$ into one liter dH_2O and adjusting the pH to 7.0 with 1N HCl.

Phosphate buffered saline (0.01M PBS) consisted of 8.0g NaCl, 0.2g KCl, 0.2g KH_2PO_4 and 0.92g Na_2HPO_4 per liter dH_2O.

An RPMI low cytotoxicity medium was used for T cell depletions and was composed of RPMI-1640 powder (10.4 g/l dH_2O), 0.3% (w/v) bovine serum

albumin (BSA--Sigma), 10 mM hepes buffer (J.T. Baker Chemical Co.) and pH was adjusted to 7.2. This BSA-supplemented medium was used to keep background killing in the presence of complement to a minimum since FCS usually contains complement-dependent cytotoxins to mouse lymphocytes. Therefore, cells were not exposed to FCS until after T cell depletion was done.

The working solution of trypan blue for viable cell counts consisted of 80% (v/v) trypan blue stock (0.2% -- Allied Chemical Corp., New York, NY) and 20% (v/v) sterile saline (4.25% NaCl).

A ficoll-hypaque solution for lymphocyte separation was prepared as follows: 12 parts of 12% (w/v) ficoll (Pharmacia Canada Ltd., Dorval, Quebec) were mixed with 5 parts of 32.8% (w/v) hypaque sodium diatrizoate (Winthrop, Aurora, Ontario) in distilled water. It was filtered and stored protected from light, at $4^{o}C$. Its density was determined to be 1.086g/ml (weight of 10ml F/H / weight of 10ml dH_2O) at $22^{o}C$, and it was prewarmed to this temperature prior to use.

Nutrient cocktail for feeding cells in culture was prepared by adding to 140ml MEM, 20ml essential amino acids (50X concentrate), 10ml non-essential amino acids (10 mM), 10ml L-glutamine (200 mM) and 2g dextrose (Fisher Scientific Co., Fairlawn, NJ). The pH was then adjusted to 5.0 with 1N NaOH and 22.6ml of 8.8% $NaHCO_3$ added to give a final pH of 7.4. The cocktail was filter sterilized, aliquoted and stored at $-20^{o}C$.

2.2.3 Lymphocyte Suspensions

Upon sacrifice of mice by cervical dislocation, spleens were removed and placed in either RPMI low cytotoxicity medium in the case of T cell depletion experiments or, otherwise, in CMEM. Single splenocyte suspensions were obtained by gentle homogenization in loose fitting glass tissue homogenizers (Fisher Sci. Co.). The cells were washed 3 times, counted by trypan blue exclusion and made up to desired concentration.

2.2.4 Culture System

One-millilitre Mishell-Dutton cultures (1967) were set up in 24-well flat-bottom tissue culture plates (Falcon 3047) at 10^7 cells per ml CMEM/well. For *in vitro* immunization, 30 ul of a 3% suspension (v/v) of sheep or ox red blood cells (SRBC, OXRBC - Woodlyn Laboratories Ltd., Guelph, Ontario) in CMEM were added per well. Cultures were incubated on a rocking platform in a 10% CO_2 atmosphere at 37°C, and each fed with 50 μl nutrient cocktail every day.

2.2.5 Preparation of T Cell Depleted Cultures

After washing and counting, CBA/J or DBA/2 lymphocytes were isolated by ficoll-hypaque separation (2,400 rpm, 20 minutes, 22°C). Spleen cells were layered in a volume ratio of 4:3 (cell suspension: F/H) over F/H and after separation, lymphocytes at the F/H:medium interface were removed by Pasteur pipette, washed 3 times in medium and counted. At this time, cells were divided into tubes labelled "not T-depleted" and "T-depleted" groups, spun down and made up to a concentration of 10^7 cells/ml in either medium alone (for "not T-depleted" group) or medium containing a mixture of anti-Thy1.2 (at 1:600) and anti-Lyt1.1 (at 1:350) monoclonal antibodies (Cedarlane Laboratories, Hornby, Ontario). The suspensions were treated on ice for 1 hour, centrifuged down, resuspended in either medium alone ("not T-depleted") or in medium containing a 1:10 dilution of Low-Tox-M rabbit complement (Cedarlane Laboratories), and incubated for 1 hour in a 37°C water bath. After 3 washes, viable cells were counted and dead cells removed by F/H separation. The remaining viable cells were removed from the interface, washed 5 times in CMEM, counted again, and diluted to desired concentration for plating.

2.2.6 Proliferation Assay

Cell proliferation as monitored by the incorporation of tritiated thymidine, was used to determine the effectiveness of the T cell depletions by responsiveness to

the T cell mitogen concanavalin A (Con A) and to lipo; olysaccharide (LPS), a B cell mitogen. This assay was also used to determine the mitogenicity of the rheumatoid factor preparations.

Cells were made up to a concentration of 5 X 10^6 cells/ml CMEM and 0.1ml/well (i.e. 5 X 10^5 cells) were plated in 96-well round bottom plates (Nunc, Denmark). Control wells were given 0.1ml of medium and the remainder were given 0.1ml of increasing concentrations (0.1μg/well up to 10.0μg/well) of either Con A (Sigma), LPS (Sigma) or RF. After 24 hours incubation in 5% CO_2 at 37^{o}C, 2uCi of ^{3}H-thymidine (New England Nuclear, Boston, Mass.) were added per well and incubated for another 18 hours. Cells were harvested on the Titertek cell harvester (Flow Labs, Rockville, MD) and ^{3}H-thymidine incorporation counted on a Beckman beta counter (Model LS3801, Beckman Instruments (Canada) Inc.). The amount of T cell depletion achieved was determined by calculating the percent inhibition of ^{3}H-thymidine uptake in the presence of Con A as compared to non T-depleted controls. The stimulation index for each group was calculated as fold increase over control.

2.2.7 Co-Stimulator Assay

RF preparations and culture supernatants were tested for the presence of IL-4 by a co-stimulator assay in the presence of a sub-optimal B cell proliferative dose of goat-anti-mouse μ chain-specific antibodies (Howard *et al.*, 1982). Using 100 μl volumes and 96-well round-bottom plates (Nunc), murine B cells (prepared by T-depletion) were plated at 2 X 10^5 cells/well in CMEM and received a sub-optimal mitogenic dose of anti-μ antibody (1 μg/ml) or increasing amounts of IL-4 from 1 ng to 4 ng/ml (20 units to 80 units/ml) (Genzyme Corp.) for a total volume of 200 μl. RF preparations at 0.3 μg/ml CMEM, or culture supernatants, were added in 100 μl volumes containing 1 μg/ml anti-μ antibody. To some wells RF was added in the presence of 20 U/ml IL-4. Proliferation was monitored as described in section 2.2.6.

2.2.8 FACS Analysis

The efficacy of the T cell depletion protocol employed was also demonstrated by fluorescence activated cell sorter (FACS) analysis. The percentage of cells expressing the following lymphocyte surface molecules was monitored: Thy 1.2 (a T cell marker), Lyt 1 (primarily a helper T cell marker but also present on a subset of B cells - the Ly 1^+ B cells) and B220 (a B cell marker).

Fifty microlitres of T depleted or non-treated cells at a concentration of 2 X 10^7 cells/ml of PBS containing 0.2% NaN_3 were added to 20 μl normal human serum (NHS) and 100 μl of diluted monoclonal rat-anti-mouse antibody (either anti-Thy 1.2 at 1:160, anti-Lyt 1 at 1:160, both from Becton Dickinson and supplied at 250 μg/ml, or anti-B220/14.8MAb at 1:100, kindly donated by Dr. S. K. Singhal). The mixtures were vortexed, incubated on ice for 30 minutes and then washed twice in PBS. To each cell pellet were added 20 μl NHS and 50 μl goat-anti-rat IgG antibody which was FITC labelled (diluted 1:75 in PBS). After vortexing and an additional 30 minute incubation on ice, the cells were washed once with PBS and resuspended in 600 μl PBS. Optimal antibody dilutions were determined using the FACS with non-treated spleen cells (data not shown). Cells with NHS only and cells with NHS plus FITC-goat-anti-rat IgG antibody were used as background controls. Fluorescence was monitored on a FACSTAR PLUS cell sorter (Becton Dickinson) interfaced with a DEC Microvax computer.

2.2.9 T Cell Factors

A T-cell derived lymphokine-containing preparation with T helper cell-replacing activity was produced according to the method of Schimpl and Wecker (1972). Two-way mixed lymphocyte cultures (MLC), containing DBA/2 (H-2^d) and CBA/J (H-2^k) spleen cells (2.5 X 10^5 cells each per well), were set up in 96-well round bottom plates (Linbro, Flow Laboratories, McLean, VA). After incubation at 37^oC for 5 days in a 5% CO_2 atmosphere, the supernatants were harvested and

centrifuged twice at 1,500 r.p.m. for 10 minutes to remove cells and debris. The supernatant was concentrated 5 fold in an "Amicon cell" (Amicon Corp., Danvers, Mass.) using a PM 10K membrane filter (Millipore Corp., Bedford, MA) under a pressure of 40 lb/in^2. After dialysis with PBS and filter sterilization, it was aliquoted and stored at -20^{o}C.

Recombinant murine interleukin 4 (rIL-4) was purchased from Genzyme Corporation, Boston, MA. It was diluted to appropriate concentration with tissue culture medium containing 10% FCS prior to use.

2.2.10 Direct Plaque Assay of Primary Antibody Responses

The numbers of IgM-anti-SRBC plaque forming cells (PFC) were determined by assaying by the method of Cunningham and Szenberg (1968) after 5 days of culture. To 150 μl of resuspended, harvested cells, were added 100 μl of 5% SRBC and 50 μl guinea pig complement (1:4 dilution, Gibco), all in BSS. This suspension was then placed in double-sided slide chambers (80 μl/chamber), sealed with wax and incubated for 1 hour at 37^{o}C. The plaques formed were then counted and expressed as the number of plaques per 10^6 viable cells recovered.

2.2.11 Immunization of Animals

Mice were immunized intraperitoneally (i.p.) with 0.1 ml of 10% SRBC in saline and sacrificed when needed for evaluation of secondary antibody responses to SRBC.

2.2.12 Plaque Assay for Secondary Antibody Responses

The numbers of total (indirect) and IgM (direct) PFCs were monitored after 3 days of culture of spleen cells from SRBC-primed mice by a modified version of Jerne's agar technique (Jerne *et al.*, 1974). To small glass tubes set up in a 42^{o}C water bath (to prevent gelling), were added 500 μl of 0.5% Sea-Plaque agarose (FMC BioProducts, Rockland, ME) in BSS, 100 μl of resuspended cells and 50 μl of SRBC (1:15 in BSS). The mixture was poured onto slides which had been

previously coated with 0.1% agarose. The slides were incubated for 1 hour at 37°C and for the measurement of indirect plaques, were inverted on slide trays containing facilitating rabbit-anti-mouse Ig serum (Cedarlane) at a 1:100 dilution in BSS, and again incubated for 1 hour at 37°C. Hemo-Lo guinea pig complement (Cedarlane) was then mixed in with the facilitating serum in the slide trays at a final dilution of 1:10. After a further 1 hour incubation at 37°C, the slide-containing trays were refrigerated for at least 1 hour prior to counting of the plaques which had developed.

In order to determine the numbers of direct or IgM PFCs, the facilitating serum step was omitted. The number of IgG PFCs was then calculated by subtracting the number of direct (IgM) PFCs from indirect (total) PFCs.

2.2.13 Heterologous Anti-Ig Antibodies

Affinity column-purified goat-anti-mouse antibodies (specific for IgG-Fc, IgG-F(ab')$_2$, or IgM, and with minimal crossreactivity to human, bovine and horse serum proteins) were purchased from Jackson ImmunoResearch Laboratories, Inc. (Avondale, PA) and diluted to desired concentrations in PBS before each use.

2.2.14 Nonspecific Immunoglobulins

Intact and F(ab')$_2$ fragments of murine IgG and human intact IgG were obtained from Jackson ImmunoResearch, and murine IgM from ICN ImmunoBiologicals (Lisle, IL), and all were diluted to desired concentrations in PBS immediately prior to use.

2.2.15 Murine Rheumatoid Factors

Purified monoclonal IgM-anti-IgG-Fc RFs were obtained as kind gifts from Drs. Coulie and Van Snick (Brussels). Three RFs (RF1, RF2 and RF5 from clones H1115A11M16, B2005E6M140 and 1307A6M80 respectively) were directed against IgG_1, and two (RF3 and RF4 from clones A6506G5M83 and A8305H6M80) against IgG_{2a}.

2.2.16 Characterization of RFs by ELISA

The enzyme-linked immunoassays employed the biotin-streptavidin procedure (Voller *et al.*, 1980; Hagen and Strejan, 1987). Washing buffer consisted of 0.01 M PBS, 0.05% Tween 20 (J.T. Baker), 0.2% gelatin (Fisher) and 0.02% Na-azide (J.T. Baker). Diethanolamine buffer (for phosphatase substrate) was made up by dissolving 0.1 g $MgCl_2 \cdot 6H_2O$ (BDH Chemicals, Toronto, Ont.), 0.2 g NaN_3 and 97 ml diethanolamine (BDH) into 500 ml ddH_2O, adjusting pH to 9.8 with 1 M HCl and bringing the volume up to 1 litre with ddH_2O (and stored at 4^oC, in the dark).

Using 100 μl volumes, microtitre wells (Falcon 3040) were coated overnight at 4^oC with either mouse IgG-Fc fragments, intact mouse IgG or IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3) purchased from Sigma. Plates were blocked for 2 hours at room temperature with 1% gelatin (Fisher) in PBS and incubated with 2-fold serial dilutions of RF samples for 1 hour at 37^oC. This was followed by biotin-labelled anti-immunoglobulin (Jackson ImmunoResearch, 1:5000 dilution, 2 hr at room temperature) then with phosphatase conjugated streptavidin (Jackson ImmunoResearch, 1 hr at 37^oC), developed for 20 minutes with p-nitrophenyl phosphate disodium (1 mg/ml diethanolamine buffer; Sigma/BDH) and the reaction was stopped with 3N NaOH, with appropriate washes between each step using washing buffer. Absorbance was read at 405 nm on an Elisa plate reader (Flow).

2.2.17 Formation of Antigen-Antibody Complexes for Suppression by Passively Administered Antibody

Intact and $F(ab')_2$ mouse IgG anti-SRBC antibody (produced previously by this lab, following multiple immunization with SRBC as described by Sinclair, 1969) was incubated with a 3% suspension (v/v) of SRBC for 15 minutes at 37^oC and then on ice for 30 minutes. Further incubations with other immunoglobulins were carried out in the same manner. All antibody concentrations used were determined

by hemagglutination tests using a 3% SRBC suspension in 96-V-well microtitre plates (Dynatech Laboratories, Inc., Chantilly, VA). The lowest dilution (highest concentration) not exhibiting hemagglutination was considered as most suitable in order that maximum immune complex formation but not overt hemagglutination could be achieved. All goat antibody and RF dilutions were determined in the presence of the suppressive Ab-Ag complexes by the same criterion (nonagglutination).

2.2.18 Statistical Methods

The significance of differences between groups was determined by analysis of variance (Zar, 1984).

2.3 RESULTS

2.3.1 Interference of suppression mediated by passively administered antibody

In order to demonstrate that the heterologous antibodies and the murine RFs used were indeed effective as IgG-Fc-blocking agents, their abilities to prevent Fc-dependent antibody-mediated suppression were determined. Hemagglutination tests were first done to determine optimal antibody dilutions for Ag-Ab complex formation. The titre of the mouse-anti-sheep erythrocyte antibody was determined to be 32 (Table 2.1) and subsequent hemagglutination tests in the presence of the Ag-Ab complex were done at a dilution of 1:64 so that excessive agglutination could be avoided. Titres for the various antibodies, controls and rheumatoid factors in the presence of sheep erythrocytes alone or as Ag-Ab complexes are shown in Table 2.1. This table also shows that the anti-Fc antibodies and the murine RFs cannot on their own agglutinate SRBCs or $F(ab')_2$-SRBC and, hence, the reconstitutive effects demonstrated are not due to binding to either the antigen or to the $F(ab')_2$ portions of immunoglobulins (or antibodies) either secreted or membrane-bound.

Table 2.2 demonstrates that when the anti-SRBC antibody was first incubated with antigen, goat anti-mouse IgG-Fc antibody and murine RF, as opposed to anti-Fab antibodies, could interfere with the suppression by the immune complexes. Since intact and $F(ab')_2$ goat antibodies specific for mouse Fc were equally effective (data not shown), no Fc-dependency of the interfering effect was demonstrated. Suppression could be prevented if the anti-SRBC antibody was incubated with either goat anti-Fc or anti-Fab *prior to* incubation with antigen. The anti-Fc antibody prevented suppression by its Fc-blocking ability and the anti-Fab exhibits the same outcome by preventing formation of the suppressive immune complex. Therefore, the inability of anti-Fab antibody to prevent suppression in the first condition is not because it, itself, is suppressive. It should be noted that

cultures receiving Ag-Ab complexes incubated with goat anti-mouse IgM exhibited no suppression although suppression was expected. In this case, the high concentration of anti-IgM used (1000-fold difference in titre compared to anti-IgG-Fc in hemagglutination test, see Table 2.1) was in keeping with the criterion for nonagglutinating but IC-forming conditions and, hence, it is probable that anti-IgM has directly stimulated the B cells via surface IgM.

All five RFs employed prevented suppression by the Ag-Ab complexes (Table 2.2) and, therefore, block IgG-Fc signals and are not immunosuppressive.

TRF interfered with the Fc-mediated suppression as demonstrated previously (Hoffman and Kappler, 1978; O'Garra *et al.*, 1987). As is shown in Table 2.2, TRF was added after 2 days of culture and was the positive control along with the antigen-stimulated group which received neither suppressive anti-SRBC antibody nor blocking agents.

2.3.2 FACS Analysis and Mitogen Responsiveness of T Cell Depleted Spleen Cells

The efficacy of the T cell depletion protocol used was examined by FACS analysis and by responsiveness to mitogens. Figures 2.1 and 2.2 give the FACS profiles of not T depleted and T depleted DBA/2 spleen cells, respectively, used in studies of primary antibody responses to SRBC. In the untreated group (Figure 2.1), 44.0% of the cells stained positive for the B cell marker B220, 36.5% were Thy 1.2 positive and Lyt 1 was found on 39.2% of cells. Thus, some non-T cells are Lyt 1 positive. This is consistent with reported findings (Hayakawa *et al.*, 1983). After T cell depletion using a mixture of anti-Thy 1.2 and anti-Lyt 1.1 and rabbit complement, the percentage of cells expressing B220 rose to 81.6 % (almost a 2-fold increase) while cells expressing Thy 1.2 and Lyt 1 dropped to 4.4% and 3.9% respectively. Because 2% of the background (goat-anti-rat FITC alone, Figure 2.2A) was excluded in defining the negative control within certain boundaries, the

percentage of T cells remaining is probably closer to 2%. The proliferative response to the T cell mitogen, Con A, was completely removed while the response to LPS, a B cell mitogen, was not affected (Table 2.3). Very similar results were obtained using spleen cells from mice primed with SRBC which were used for secondary responses. T cell depletion resulted in an enrichment for B220-positive cells while cells expressing Thy 1.2 and Lyt 1 dropped to only 1.7% and 1 6% respectively as shown in figures 2.3 and 2.4. The mitogen response to ConA was again completely obliterated while the LPS response remained, and in fact, was slightly elevated (Table 2.3).

2.3.3 Reconstitution of T-Depleted CBA/J Splenocyte Cultures by RF in a Primary T Dependent Response

The reconstitutive ability of RF was tested in the response of T cell-depleted spleen cells to a T-dependent antigen (SRBC) *in vitro*. Three experiments were carried out using four RF preparations, and these experiments were combined (Figure 2.5). Murine RF was added at the start of the cultures, as was the goat anti-mouse IgG-Fc antibody. A T-cell-replacing factor supernatant (TRF) given at 2 days, the time at which it is normally active (Schimpl and Wecker, 1972; Sinclair and Panoskaltsis, 1986a), served as the positive control. Cultures given none of the above, and either stimulated or not stimulated with antigen, were also included as negative controls. It can be seen that these latter cultures were indeed suppressed compared to the positive TRF control. There was a dose-dependent rise in the response when RF was added. The PFC response in the presence of goat anti-mouse IgG-Fc antibody was low. This was due to the fact that the dose of anti-Fc antibody used was shown, in later experiments, to be less than that necessary for full reconstitution.

These experiments demonstrate that RF can reconstitute a T-deficient culture; this complements previous results demonstrating reconstitution by goat

antibodies against murine IgG-Fc (Sinclair and Panoskaltsis, 1986a; 1986b; 1987a; 1987c).

The experiments presented in figure 2.5 combined all RFs, irrespective of IgG subclass specificity, since they were equally effective at reconstitution.

2.3.4 Requirement for T depletion and antigen stimulation

In keeping with the prediction that murine RF would exhibit similar reconstituting characteristics as the heterologous anti-Fc antibodies used previously (Sinclair and Panoskaltsis, 1987c), the conditions under which the augmenting effect could be demonstrated were examined. Table 2.4 shows that the effect of RF4 is seen under the same conditions as those determined for goat anti-mouse IgG-Fc antibody; that is, in cultures which are T-depleted and antigen-stimulated. Furthermore, RF4 was more potent than goat anti-mouse Fc antibody in reconstituting the response of a T-depleted culture to a T-dependent antigen.

2.3.5 Antigenic Specificity of Reconstitution

In order to demonstrate that the reconstitutive effect of RF was specific for the immunizing antigen and did not result in a polyclonal-type of activation, splenic B cells were immunized *in vitro* with erythrocytes from one of two different species (sheep and ox) and plaque-forming assays with each antigen were performed in parallel. As can be seen from Figure 2.6, reconstitution of the anti-SRBC antibody response was only seen when cultures were immunized with SRBC and not with OXRBC and SRBC immunization did not affect the anti-OXRBC response. The reverse conditions were also true and confirmed the antigenic specificity of the reconstitution. Therefore, RF can only block Fc signals being delivered by Ag-Ab complexes on the surface of the Ag-specific B cell and does not act in a non-antigen-directed manner. Figure 2.7 also reaffirms the fact that RF must be present from culture initiation since it does not significantly affect the response above background levels when added at 48 hours of culture

2.3.6 RF does not induce proliferation

To further rule out any mitogenic properties of the RF preparations used, proliferation of murine spleen cells, as measured by ^{3}H-thymidine uptake, in response to the RFs was determined. Table 2.5 shows that RFs in amounts that cause reconstitution of the Ab response have no mitogenic activity. In contrast, TRF causes a 2-fold increase in ^{3}H-thymidine uptake which reaffirms the fact that RF and TRF operate through different mechanisms.

SRBC also did not cause an increase in the proliferative response since the SRBC-specific population is only a fraction of the total. Hence, those agents which were "antigen-directed" differed from those which were not, such as TRF, Con A, and LPS, which stimulate cells via routes other than the surface antigen receptor.

2.3.7 Abrogation of RF effect by nonspecific mouse IgG

The Fc-blocking ability of heterologous goat anti-mouse Fc antibodies was specific for only those antibodies which could bind the Fc portion of murine IgG endogenously formed in the culture (Sinclair and Panoskaltsis, 1987c). A comparable experiment involving RF could not be carried out with the RFs available. Therefore, the ability of intact murine IgG to interfere with reconstitution was studied. To this end, nonspecific murine IgG was added at culture initiation to T-depleted cultures which were given a reconstituting dose of RF4. The reconstitutive effect of RF4 was abrogated in a dose-dependent fashion specifically by intact murine IgG and not by the F(ab')$_2$ fragment or by murine IgM (Figure 2.8). The abrogation obtained by the addition of human IgG is most likely due to cross-reactivity. This experiment also lays to rest the possibility that immunostimulatory complexes were generated as described previously (Morgan and Weigle, 1983) as opposed to simple blockade of negative Fc signals, since the abrogation of the RF effect by only intact IgG indicates that the IgG has bound to the RF.

Thus, the augmenting effect of RF4 is due to its ability to bind to the Fc portion of murine IgG on the stimulating antigen and block negative Fc signals, and not due to the formation of stimulatory immune complexes.

2.3.8 RF specific for IgG_{2a} is more effective at reconstitution than RF against IgG_1 when less T-cell help is available

In many instances where profound T-cell depletion was achieved, as indicated by the low antigen control and lack of response to Con A, only RF with specificity for IgG_{2a} was able to reconstitute the response (Table 2.6 and Figure 2.9). Of the two anti-IgG_{2a} RFs, RF4 showed superior reconstituting ability. Thus, either RF4 has the highest affinity for that portion of the Fc which mediates suppression in this system, compared to the other RFs, or IgG_{2a} is more potent than IgG_1 at immunosuppressing. Since all RFs were produced in the same way by one laboratory (Van Snick and Coulie, 1983; Coulie and Van Snick, 1985) and diluted by the same buffer, there is no reason to believe that RF4 would exhibit some occult reconstituting ability other than by blocking negative Fc signals.

The reactivity patterns of the 5 RFs as determined by ELISA and expressed as fold increase over background (Table 2.7) also show that RF3 and RF4 have specificity for determinant(s) not recognized by the other RFs in as exclusive a manner since RF1 and RF2 show extensive polyspecificity. Since the other RFs can also recognize IgG_{2a} at least to some degree, the difference may be due to the avidity of RFs for the IgG with RF4 having highest avidity for that epitope (carbohydrate?) on IgG which is responsible for delivery of the negative signal. This may account for its superior reconstituting abilities. Because of its greater potency in reconstitution, RF4 was used in the majority of the experiments.

2.3.9 Synergism between RF and a late-acting T-cell replacing factor

As stated above, it was observed that, in some cases of profound T-cell depletion, RFs1-3 and 5 could not reconstitute the response. A mixture of all four

nonreconstituting RFs in a single administration at culture initiation could slightly increase the response (Table 2.8, line 5 as opposed to line 2) and this response markedly increased if a marginally effective dose of TRF (Table 2.8, line 4) was also added on Day 2 (Table 2.8, line 6). Thus, synergism between RF and TRF was demonstrated. A late-acting B-cell differentiation factor(s) may be needed for full scale antibody production to be detected by the methods we have used.

Alternatively, RF added only on Day 0 may be broken down in culture (being an IgM antibody as opposed to a heterologous goat IgG anti-mouse Fc), and, since it had been previously demonstrated (Sinclair and Panoskaltsis, 1987c) that increasing amounts of anti-Fc antibody are needed during the first 3 days of culture, it was worth determining what effect the addition of all four RFs during each of the first 3 days would have. It was seen that addition of RFs on each of Days 0, 1, and 2 resulted in good reconstitution of the response (Table 2.8, line 7) when compared to a single administration on Day 0 (Table 2.8, line 5) or on Day 2 (Table 2.8, line 8). Table 2.8, line 8 also served as a negative control since it was previously shown (Sinclair and Panoskaltsis, 1986a; 1987c) that the Fc-binding agent must be present from culture initiation.

Since TRF is not even necessary when Fc signals are blocked effectively during the first three days of culture (Table 2.8, line 7), the demonstration of synergy between RF and TRF may be beside the point. It actually demonstrates that the rate-limiting role of late-acting TRFs may also be related to the attenuation of negative Fc signals.

2.3.10 Reconstitution by RF is not Mediated by Interleukin 4

Because IL-4 has been shown to interfere with Fc-mediated immunosuppression (O'Garra *et al.*, 1987; Phillips *et al.*, 1988; Laszlo and Dickler, 1988), it was important to demonstrate that the RF preparations used and the supernatants from RF-reconstituted cultures did not contain IL-4. IL-4 is mitogenic

for B cells and is synergistic in its mitogenicity in the presence of sub-optimal mitogenic doses of anti-μ antibody (Howard *et al.*, 1982). It can be seen in Table 2.9 that this co-stimulator assay is effective on murine B cells using goat-anti-mouse μ Ab in the presence of recombinant murine IL-4. No IL-4 activity could be demonstrated in either RF4, which was the most potent at reconstitution, or in the supernatants of cultures which had been reconstituted with RF4. RF4 also did not have anti-μ activity as demonstrated by the lack of synergistic action in the presence of IL-4.

2.3.11 Reconstitution of $?^0$ T-Dependent Response

RF was also able to reconstitute the secondary anti-SRBC IgG response of T cell-depleted spleen cells taken from SRBC-primed DBA/2 mice (Table 2.10) in a dose-dependent fashion (Figure 2.10). However, as noted previously in the literature (Schimpl and Wecker, 1973), the secondary response of T-depleted primed B cells is difficult to reconstitute with TRF and usually only reaches 1/3 of the antigen response of untreated spleen cells. Since the RF-reconstituted response is compared to the TRF-reconstituted level which is always higher, it should be stated that reconstitution by RF was not always as dramatic as one would like and certainly not as effective as in the primary response. Hence, only 3 of 5 experiments are presented. The difficulty in attaining reconstitution is likely due to the abundance of IgG antibody in these cultures and having demonstrated that the reconstitution by RF can be easily abrogated with non-specific IgG (Figure 2.8), it is not difficult to propose that the RF is quickly consumed in immune complexes composed of IgG and SRBC (the antigen) (or maybe even to surface IgG) since RF has a higher avidity for complexed IgG than for free IgG. It is likely that using higher doses of RF to make up for this will result in more dramatic reconstitution but those experiments could not be done due to the limited supply of RF. However, it is also an interesting aside that the ability to reconstitute T-depleted cultures was

inversely related to the level of the IgG response of the untreated (not T-depleted) controls. The IgM responses of T-depleted spleen cells from SRBC-primed mice were not reconstituted with either RF or TRF (Table 2.10). However, this not surprising as these responses were monitored after only three days of culture and the IgM response was found to peak on day 5. The observation of limited but significant reconstitution of the IgG response when RF was added at 48 hours (day 2), as opposed to culture initiation, demonstrates that blocking later-occurring negative Fc signals can still rescue some of these primed cells.

TABLE 2.1

Determination of Optimal Ag-Ab Complex Formation Conditions
Hemagglutination Tests

Antibody	SRBC only	SRBC-Ab Complex[a] Intact	$(Fab')_2$
Mouse IgG-anti-SRBC	64[b]	-	-
Mouse $F(ab')_2$-anti-SRBC	128	-	-
Goat IgG-anti-mouse-Fc 1.8mg/ml	0	1600	0
Goat $F(ab')_2$-anti-mouse-Fc 1.4mg/ml	0	1600	0
Goat IgG-anti-mouse-$F(ab')_2$ 2.1mg/ml	0	400	1600(±)[c]
Goat $F(ab')_2$-anti-mouse-$F(ab')_2$ 1.8mg/ml	0	400	1600(±)
Goat IgG-anti-mouse-IgM (μ) 1.6mg/ml	0	10	0
Normal Mouse Serum	0	10(±)	10(±)
RF1 9.0mg/ml	0	300	0
RF2 6.5mg/ml	0	100(±)	0
RF3 3.5mg/ml	0	270(±)	0
RF4 3.5mg/ml	0	100(±)	0
RF5 4.0mg/ml	0	100(±)	0

[a]SRBC + Intact mouse IgG-anti-SRBC @1:128 or $F(ab')_2$fragment @1:256.
[b]Titre at end point of hemagglutination.
[c](±) = marginal agglutination at that dilution.

TABLE 2.2

Antibody-Induced Suppression
Interference by Anti-Fc Antibody and Rheumatoid Factor

Group	PFC/million ± SE (n)
No antigen	34.8 ± 4.8 (12)
Antigen (SRBC)	339.3 ± 38.9 (12)
+ TRF[b]	620.1 ± 21.1 (4)
Antigen-F(ab')$_2$-anti-SRBC complex[a]	294.5 ± 24.2 (6)
+ TRF[b]	627.7 ± 34.5 (6)
+ Anti-Fc[c]	335.0 ± 34.6 (4)
Antigen-anti-SRBC complex[a]	110.2 ± 15.7 (14)
+ TRF[b]	304.8 ± 51.4 (10)
+ Anti-Fc[c]	307.8 ± 30.3 (12)
+ Anti-Fab[c]	143.2 ± 5.9 (6)
+ Anti-IgM[c]	253.0 ± 30.7 (4)[d]
+ Anti-IgM (1/100)	158.0, 358.8
+ NMS	300.5 ± 29.5 (4)
+ NMS (1/100)	86.8, 166.1
+ RF1[e]	283.5 ± 20.6 (6)
+ RF1 (1/10)	304.9, 239.0
+ RF1 (1/100)	137.4, 100.3
+ RF2[e]	250.7 ± 16.0 (6)
+ RF3[e]	284.8 ± 28.1 (6)
+ RF4[e]	259.1, 389.5
+ RF4 (1/100)	184.3, 181.7
+ RF5[e]	292.3 ± 41.2 (4)
Anti-SRBC antibody	
+ Anti-Fc (then Ag)[f]	201.7, 250.0
+ Anti-Fab (then Ag)[f]	241.2, 291.4

[a]Ag-Ab complex added at culture initiation of T cell-non-depleted CBA/J splenocytes.
[b]TRF (reconstituting dose for T-depleted cultures) added on Day 2.
[c]Goat anti-mouse antibodies were first incubated with the Ag-Ab complex and the resulting mixture was added at culture initiation (Day 0).
[d]Where n=2, the two experimental values are given.
[e]RFs were incubated with the Ag-Ab complex prior to addition to culture on Day 0.
[f]Goat anti-mouse IgG antibodies were first incubated with the suppressive anti-SRBC Ab and then with the Ag before addition to culture on Day 0.

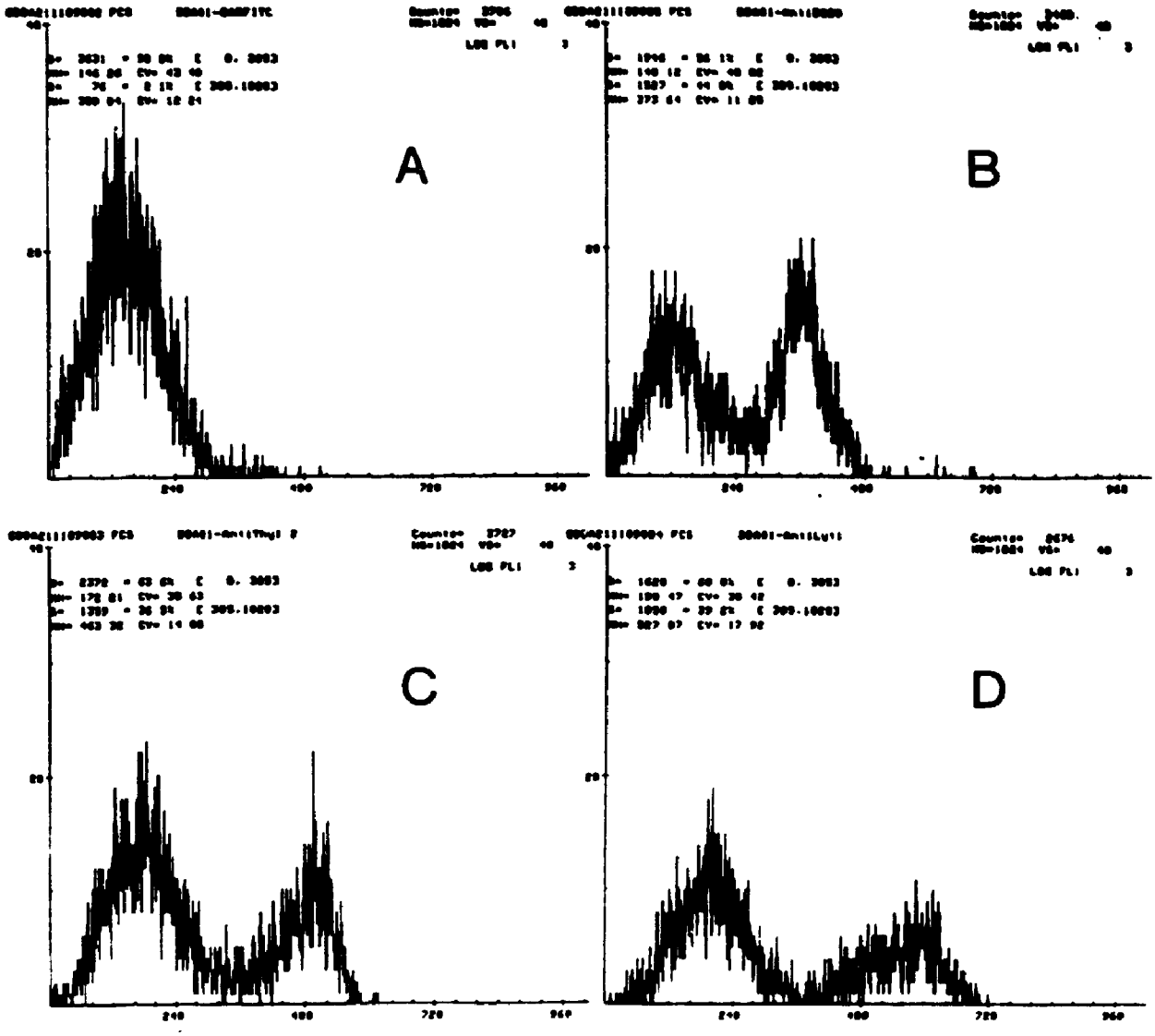

Figure 2.1. FACS profiles of T cell non-depleted, not primed DBA/2 spleen cells in presence of FITC-labelled goat-anti-rat IgG alone (A, background); rat-anti-mouse B220 (14.8MAb) (B); rat-anti-mouse Thy 1.2 (C); and rat-anti-mouse Lyt 1 (D). Autofluoresence of cells in presence of PBS alone did not significantly differ from A.

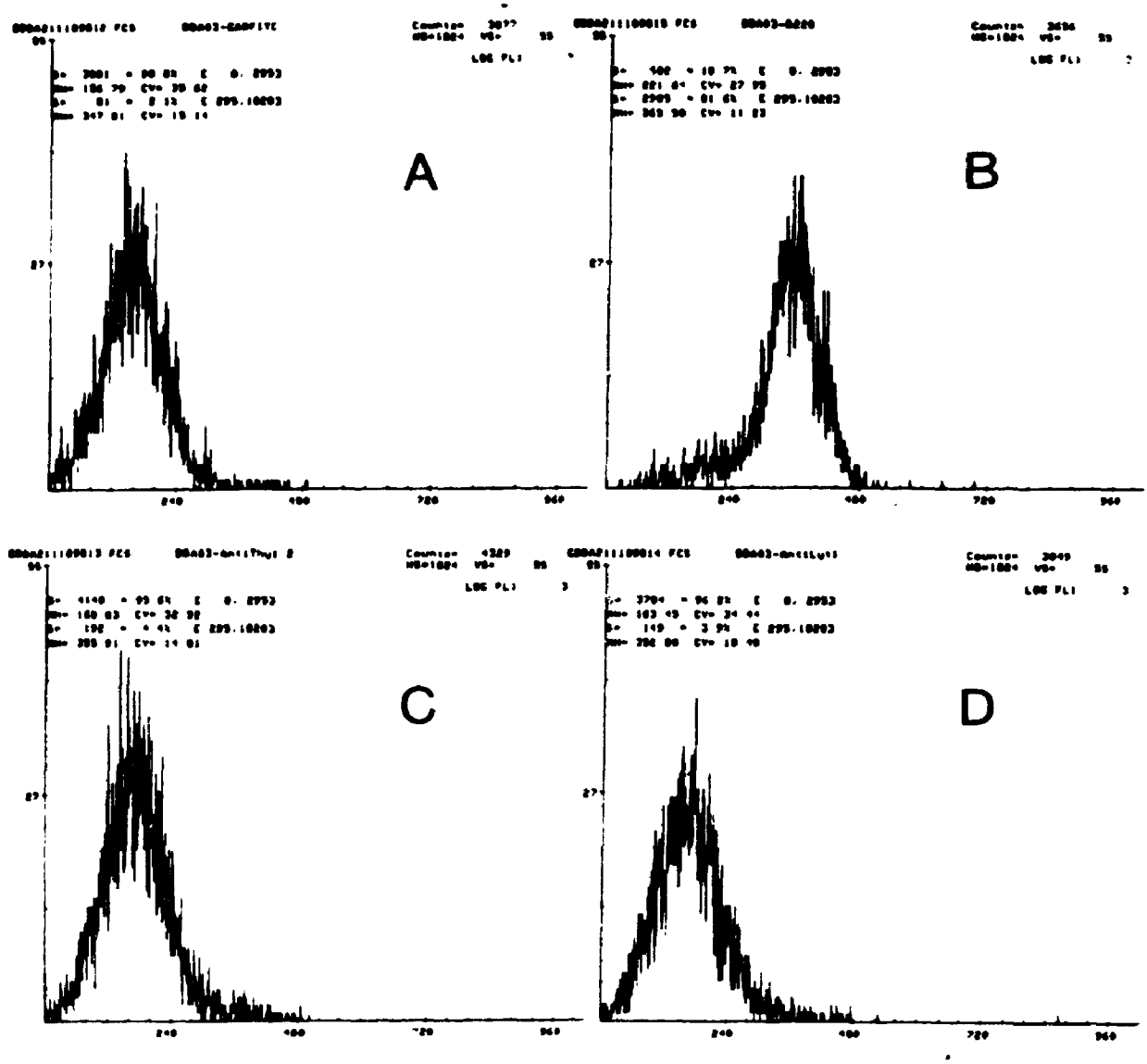

Figure 2.2. FACS profiles of T cell-depleted, not primed DBA/2 spleen cells. Descriptions of A, B, C and D as per Figure 2.1.

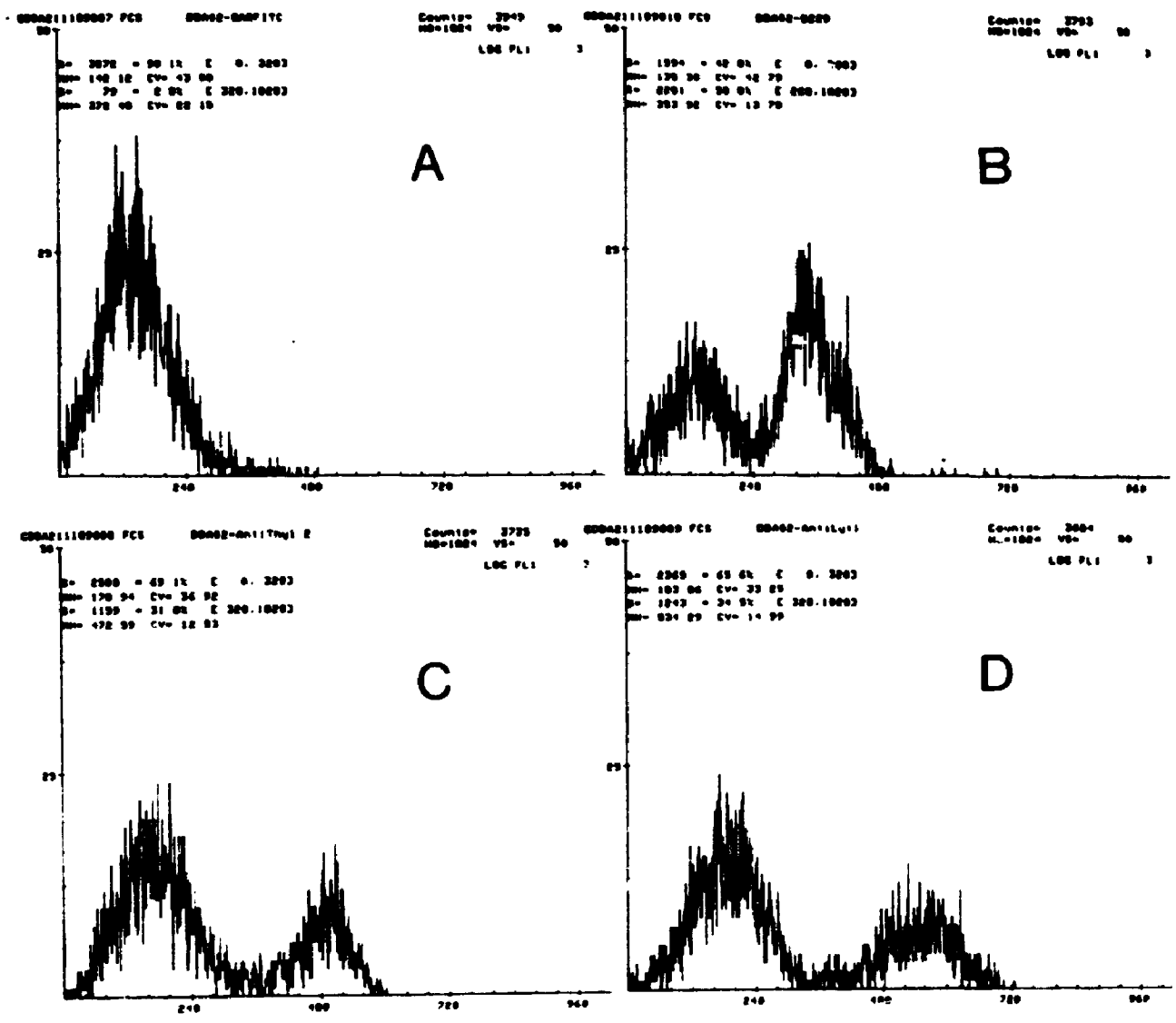

Figure 2.3. FACS profiles of T cell non-depleted, SRBC-primed DBA/2 spleen cells. Descriptions of A, B, C and D as per Figure 2.1.

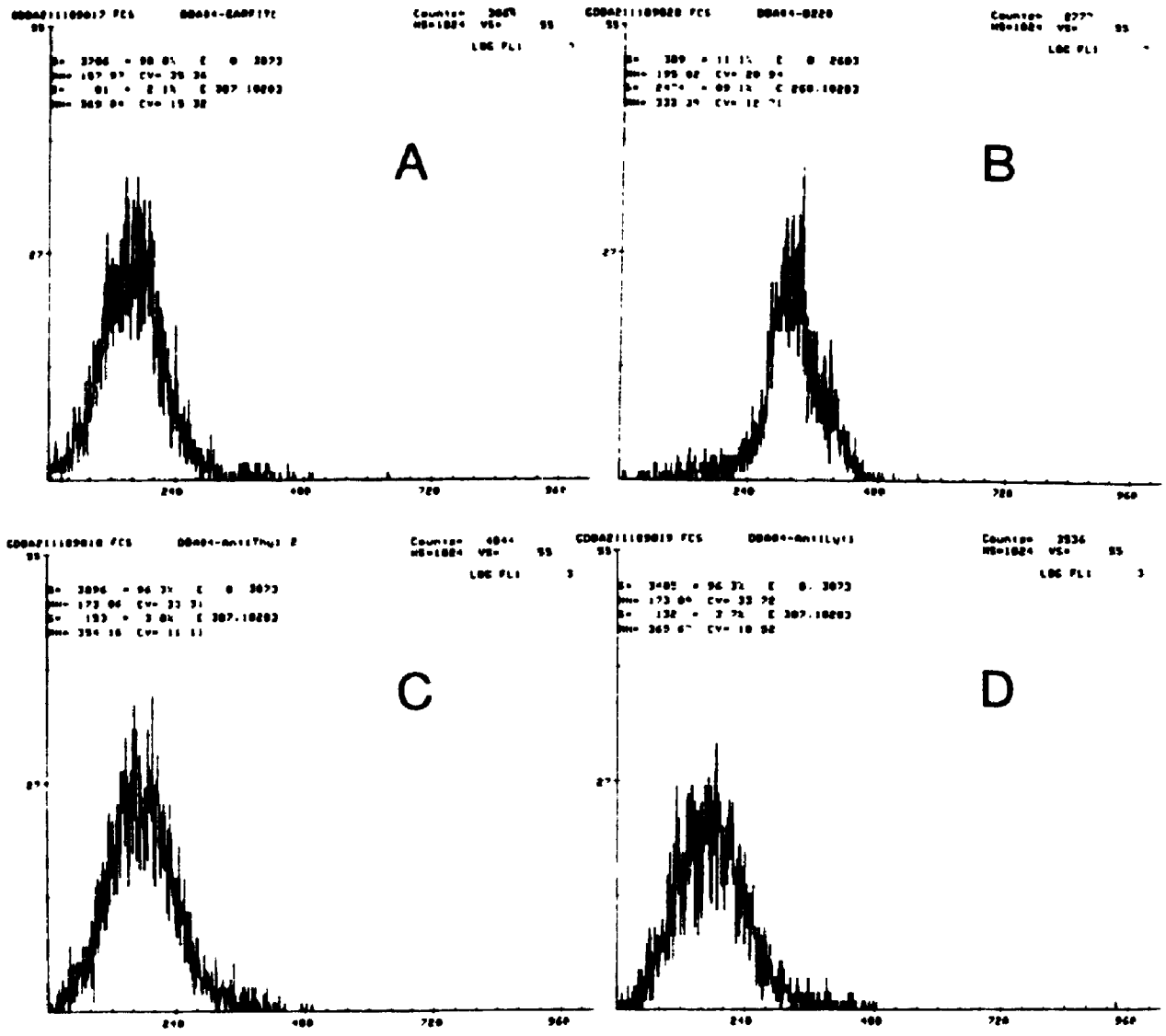

Figure 2.4. FACS profiles of T-cell-depleted, SRBC-primed DBA/2 spleen cells. Descriptions of A, B, C and D as per Figure 2.1.

TABLE 2.3
Effect of T cell Depletion on Mitogen-Induced Proliferation

Group	Not T-Depleted	T-Depleted
Non-Primed Cells +		
Medium (Control)	3123 ± 136 (4)[a]	3259 ± 178 (4)
Concanavalin A[b]	172258 ± 12447 (4)	2098 ± 181 (4)
Lipopolysaccharide[c]	15280 ± 628 (4)	14272 ± 2352 (4)
SRBC-Primed Cells +		
Medium (Control)	6243 ± 1087 (3)	2462 ± 181 (4)
Concanavalin A	169796 ± 12131 (4)	1878 ± 263 (4)
Lipopolysaccharide	35238 ± 3916 (4)	15671 ± 1907 (4)

[a]Mean uptake of ^{3}H-thymidine in cpm/minute ± standard deviation (number in group).
[b]0.25 μg ConA per well/5 X 10^5 cells (optimal dose).
[c]0.5 μg LPS per well (optimal dose).

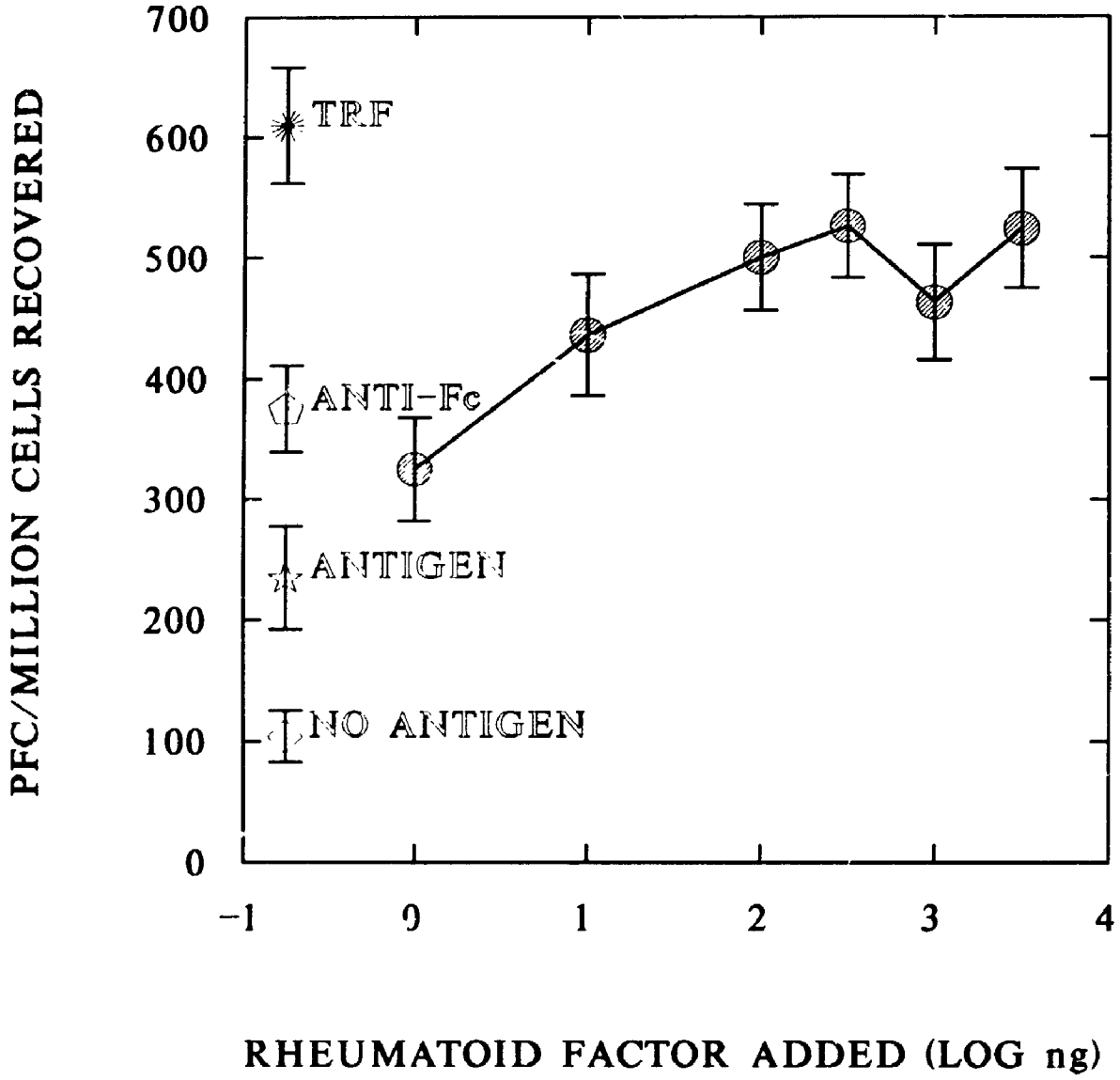

Figure 2.5. Reconstitution of T-cell-depleted cultures with murine IgM rheumatoid factor. T-depleted cultures were either not stimulated (no antigen) or stimulated (antigen) with sheep erythrocytes. Antigen-stimulated cultures received rheumatoid factor (circles) at the indicated doses, T-cell-derived lymphokine-containing supernatants (TRF), or goat-anti-mouse IgG-Fc antibody (anti-Fc). The number of cultures per group ranged from 10 to 12 and the combined results of three experiments are presented. Vertical bars indicate standard error of the mean.

TABLE 2.4

Reconstitution of T Cell Depleted, Antigen-Stimulated Cultures by Fc Signal Blockade

Groups	Normal	T-Depleted
No antigen	31.9 ± 8.1 (11)[a]	30.5 ± 5.9 (10)
+ RF4 (300 ng)[b]	33.5 ± 9.2 (13)	27.8 ± 4.1 (6)
+ Anti-Fc (300 ng)[b]	47.1 ± 12.8 (7)	40.8 ± 7.9 (6)
+ TRF[c]	66.5 ± 16.5 (7)	63.9 ± 13.5 (6)
Antigen[d]	297.5 ± 19.2 (12)	45.1 ± 48.9 (10)
+ RF1 (300 ng)[b]	280.3 ± 41.3 (2)	98.3 ± 66.4 (2)
+ RF2 (300 ng)[b]	288.0 ± 24.5 (2)	87.3 ± 41.8 (2)
+ RF3 (300 ng)[b]	306.4 ± 22.4 (4)	160.5 ± 48.5 (6)
+ RF4 (300 ng)[b]	235.5 ± 13.4 (14)	209.9 ± 49.3 (6)
+ Anti-Fc (300 ng)[b]	265.5 ± 33.5 (8)	165.6 ± 41.1 (6)
+ TRF[c]	399.9 ± 35.2 (12)	339.9 ± 35.9 (10)

[a]PFC/10^6 viable cells recovered ± SE (n).
[b]Added at culture initiation (Day 0).
[c]Reconstituting dose added on Day 2.
[d]Sheep red blood cells added at culture initiation (Day 0).

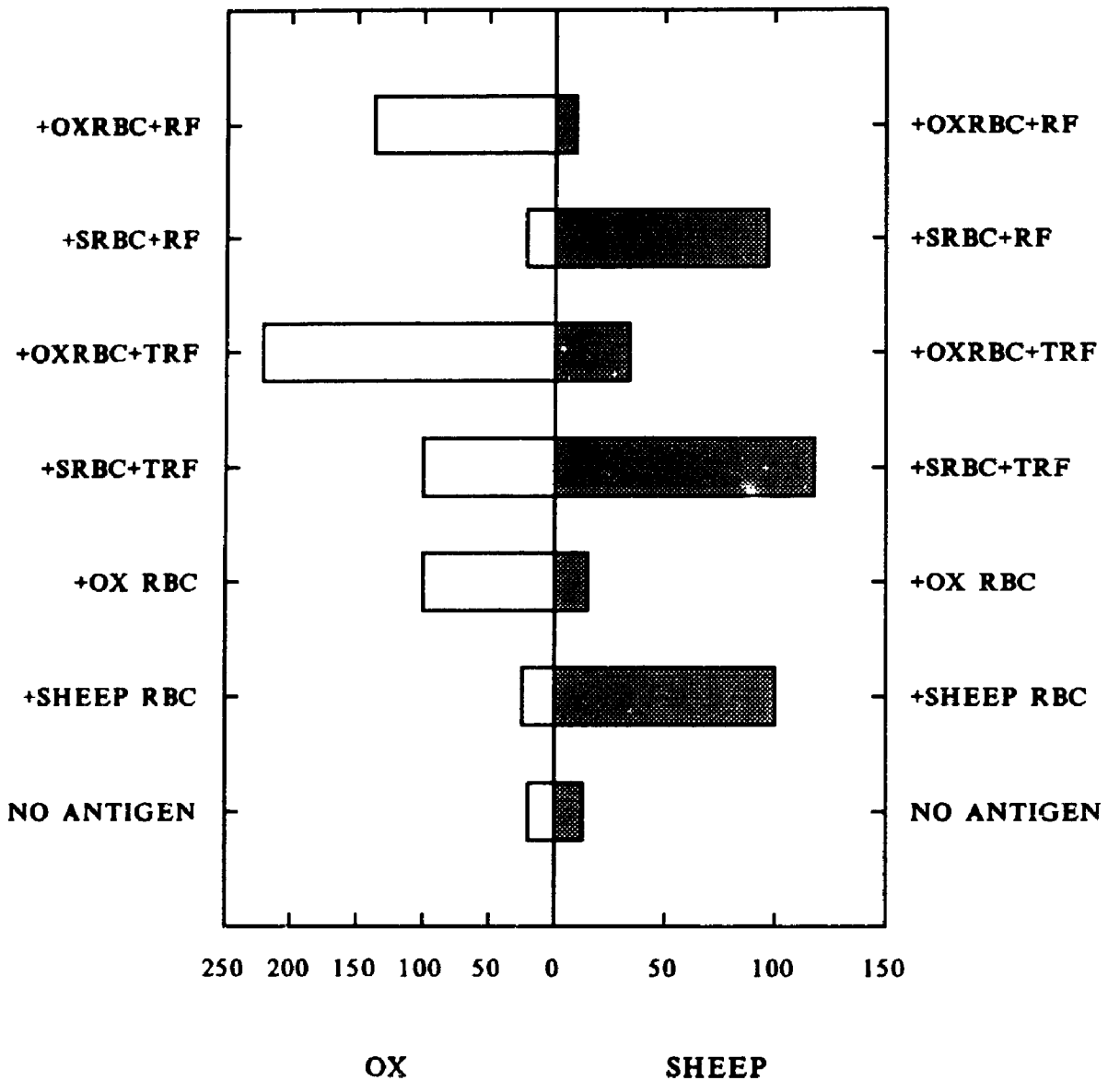

Figure 2.6. Antigenic specificity of anti-SRBC response and lack of RF effect in the presence of T cells. The left hand side of the figure (dotted bars) shows the response against ox red blood cells and the anti-SRBC response is depicted on the right (hatched bars). Along the Y-axis are listed the culture conditions versus the percent of the control response (X-axis). The positive control response for each antigen was set at 100%. RF3 was added at a dose of 0.3 μg/culture containing 10^7 DBA/2 spleen cells at culture initiation. TRF was added at 48 hours of culture at a dose equivalent to that needed for reconstitution of T-depleted cells.

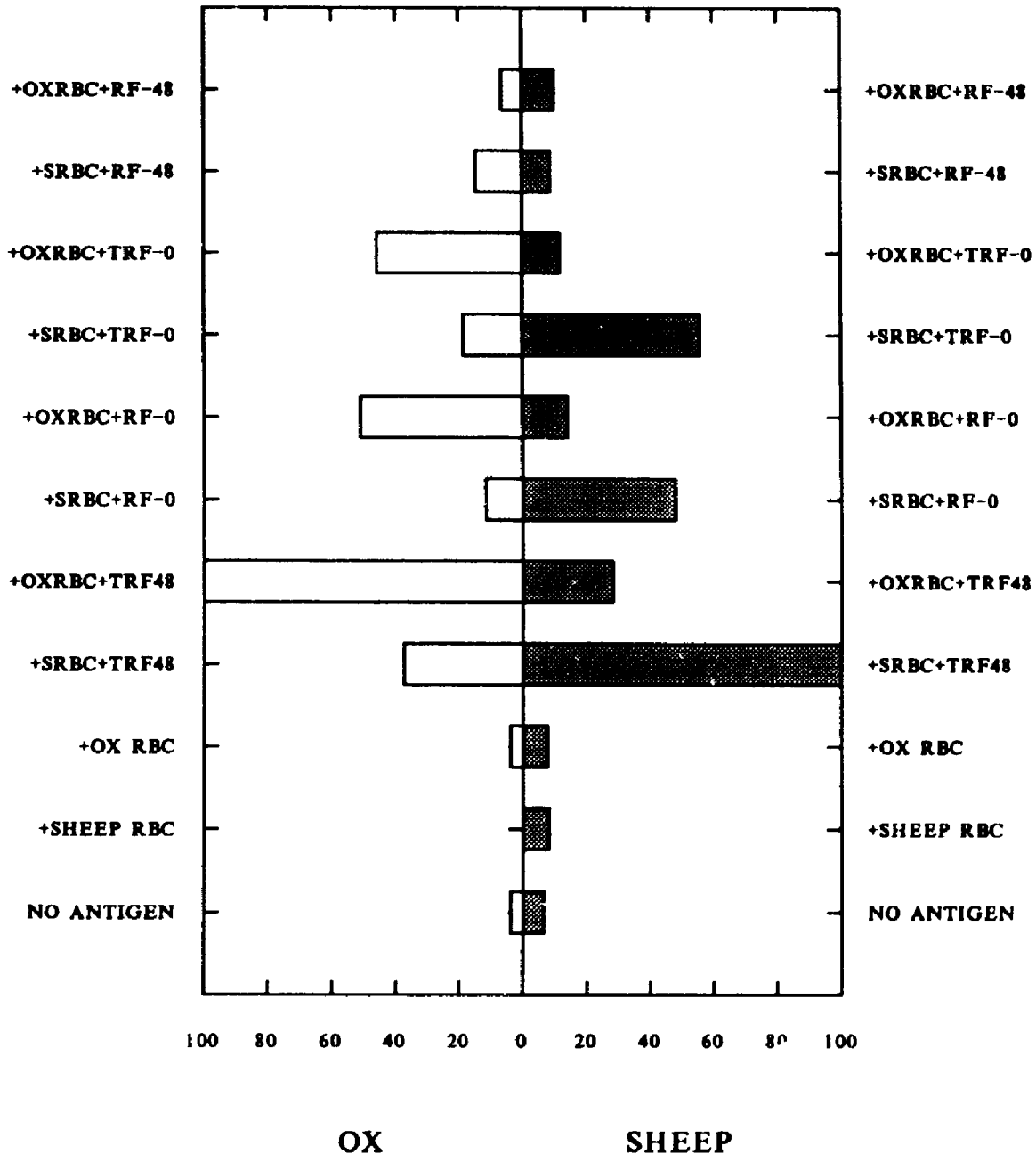

Figure 2.7. Antigenic specificity of the reconstitution of the T-dependent antibody response of T-depleted DBA/2 spleen cells by RF3. The figure is set up as per Figure 2.6. The times of RF or TRF addition are indicated. The positive control response for each antigen (corresponding antigen with addition of reconstituting dose of TRF added at 48 hours) was set at 100%.

TABLE 2.5

RF Does not Induce Proliferation of Murine Spleen Cells

Group	Not T-Depleted	T-Depleted
Not primed cells[a] +		
Con A	21.23 ± 4.01 (20)[b]	1.20 ± 0.13 (20)
LPS	4.20 ± 0.11 (19)	4.40 ± 0.25 (20)
TRF	2.10 ± 0.15 (4)	1.90 ± 0.05 (4)
RF1	0.91 ± 0.05 (4)	0.84 ± 0.02 (4)
RF2	0.91 ± 0.09 (4)	0.90 ± 0.05 (4)
RF3	1.10 ± 0.07 (8)	1.20 ± 0.10 (8)
RF4	1.00 ± 0.02 (8)	1.00 ± 0.04 (8)
RF5	0.97 ± 0.05 (4)	0.86 ± 0.02 (4)
SRBC	0.96 ± 0.03 (8)	1.00 ± 0.05 (8)
SRBC-Primed Cells[c] +		
Con A	18.52 ± 2.03 (16)	1.00 ± 0.13 (16)
LPS	4.30 ± 0.26 (15)	5.10 ± 0.28 (15)
TRF	1.70 ± 0.10 (8)	2.10 ± 0.10 (8)
RF1	1.10 ± 0.05 (4)	0.96 ± 0.02 (4)
RF2	1.10 ± 0.05 (4)	0.95 ± 0.03 (4)
RF3	1.10 ± 0.04 (8)	1.20 ± 0.07 (8)
RF4	0.97 ± 0.03 (4)	0.91 ± 0.02 (4)

[a]Control c.p.m. for non-primed cells: 20,108 ± 2,509 (19), non-T-depleted; 19,269 ± 2,200 (19), T-depleted.
[b]Expressed as mean stimulation index over control ± standard error (n).
[c]Control c.p.m.: 22,513 ± 4,049 (15), non-T-depleted; 15,800 ± 3,034 (15), T-depleted.

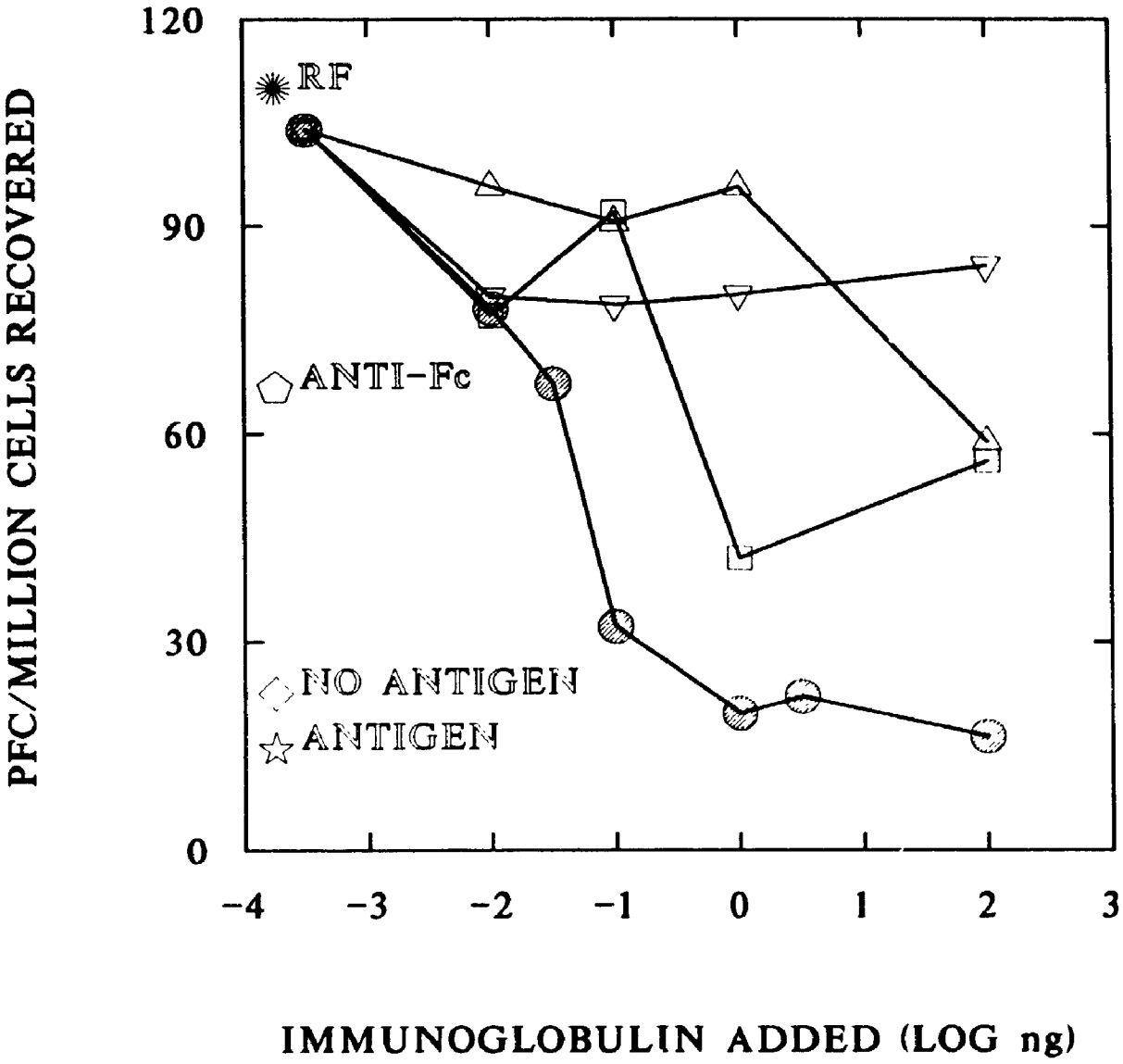

Figure 2.8. Abrogation of RF reconstitution by nonspecific intact IgG. Controls: 300 ng RF4, 300 ng goat-anti-mouse IgG-Fc added to T-depleted cultures at culture initiation with antigen (sheep erythrocytes). T-depleted culture response with ☆ or without (◇) antigen, in the absence of reconstituting RF or goat-anti-Fc. Antigen-stimulated and RF-reconstituted cultures received increasing doses of intact mouse IgG ▨), mouse F(ab')2 of IgG (/), intact mouse IgM ( ), or intact human IgG ( ). The combined results of three experiments are shown. RF (*) was about 50% of the TRF-reconstituted response.

TABLE 2.6

Comparison of Reconstituting Ability of Five Monoclonal Rheumatoid Factors

Group		PFC/10^6 cells ± SE (n)	p-value[a]	
No Antigen		29.5 ± 5.4 (21)		
Antigen[b]		28.4 ± 4.2 (22)		
+ TRF	Day 2	268.7 ± 17.8 (20)	0.000	
+ GαMFc	Day 0 (1 μg)	106.5 ± 22.4 (12)	0.000	
+ RF1[c]	Day 0 (300 ng)	32.4 ± 7.9 (8)	0.9	
+ RF2[d]	Day 0 (300 ng)	39.0 ± 5.2 (12)	0.6	
+ RF3[e]	Day 0 (300 ng)	75.0 ± 11.1 (14)	0.012	0.003[h]
+ RF4[f]	Day 0 (300 ng)	133.7 ± 15.2 (14)	0.000	
+ RF4	Day 2 (300 ng)	20.1 ± 6.8 (4)		
+ RF5[g]	Day 0 (300 ng)	44.5 ± 2.8 (2)		
+ MIgM	Day 0 (300 ng)	27.5 ± 6.9 (4)		
+ MIgG	Day 0 (300 ng)	20.0 ± 0.7 (4)		
+ MF(ab')$_2$	Day 0 (300 ng)	26.1 ± 5.2 (4)		
+ HIgG	Day 0 (300 ng)	23.4 ± 1.8 (4)		

[a]Compared to antigen control group by analysis of variance.
[b]Sheep red blood cells added at culture initiation (Day 0).
[c]Clone No. H1115A11M16 IgMαIgG$_1$.
[d]Clone No. B2005E6M140 IgMαIgG$_1$.
[e]Clone No. A6506G5M83 IgMαIgG$_{2a}$.
[f]Clone No. A8305H6M80 IgMαIgG$_{2a}$.
[g]Clone No. 1307A6M80 IgMαIgG$_1$.
[h]By analysis of variance and multiple range tests comparing RFs1-4 as a group against antigen control.

Also by analysis of variance and multiple range tests:

Comparison	p-value
RF4 vs GαMFc	0.2
RF4 vs TRF	0.000
RF4 vs RF3	0.004

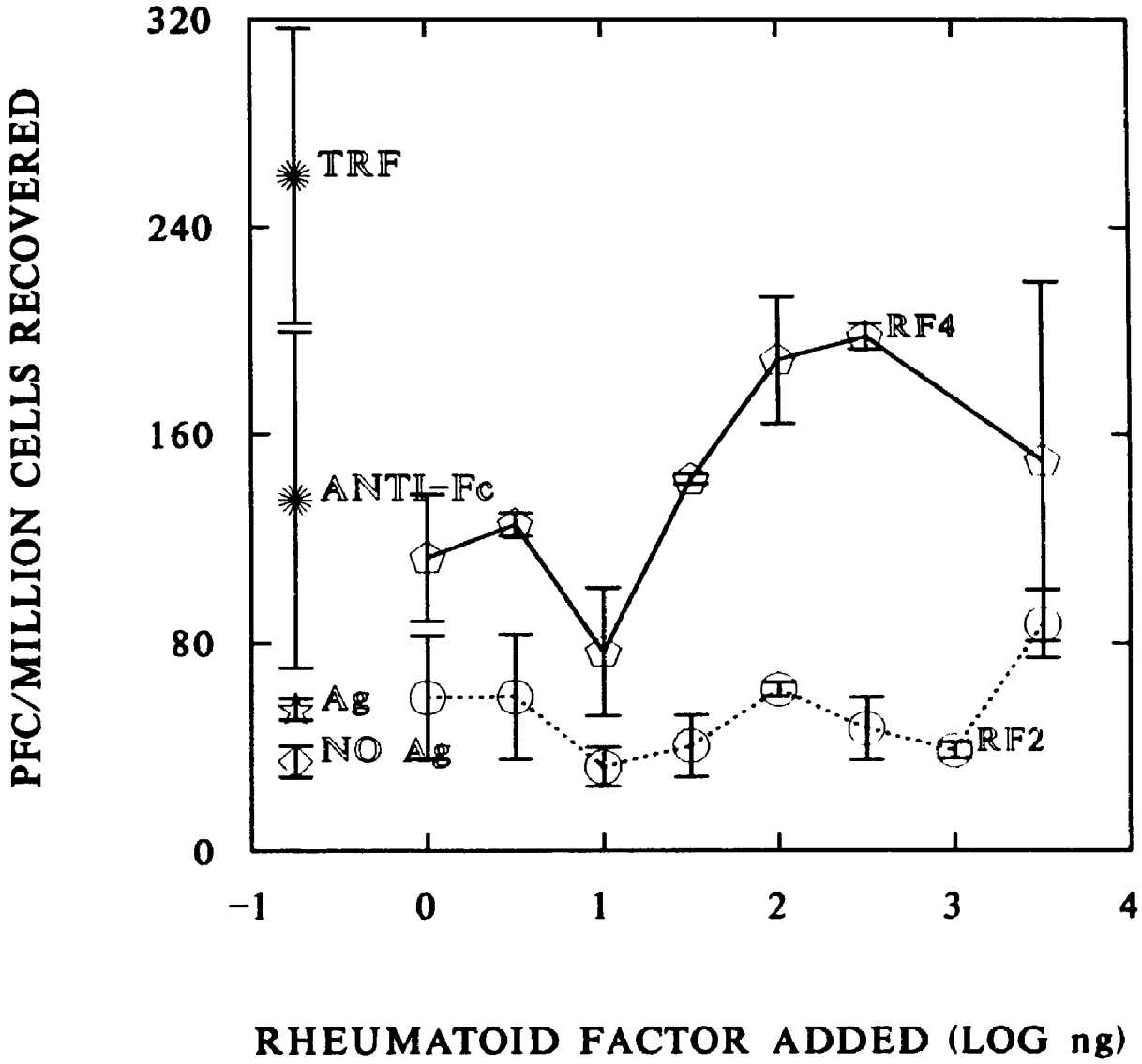

Figure 2.9. Dose-response comparison of reconstituting ability of two monoclonal rheumatoid factors. T-depleted CBA/J spleen cell cultures were either not stimulated (no antigen) or stimulated (Ag) with sheep erythrocytes. Antigen-stimulated cultures received rheumatoid factor 4 (anti-IgG_{2a}) (hexagons) or RF2 (anti-IgG_1) (circles) at the indicated doses, T-cell-derived lymphokine-containing supernatants (TRF), or 100 ng goat-anti-mouse IgG-Fc antibody (anti-Fc). Vertical bars indicate standard error of the mean.

TABLE 2.7

Reactivity Patterns of 5 Monoclonal RFs

	Binding[a] to						
	Fc	IgG	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	ssDNA
RF1	3.6[b]	4.2	2.8	3.7	<2	3.5	2.5
RF2	20.4	19.6	5.9	7.2	3.4	14.9	7.3
RF3	2.6	4.2	<2	11.3	<2	3.9	<2
RF4	<2	7.3	<2	15.1	2.2	<2	<2
RF5	<2	<2	2.0	<2	2.0	<2	<2

[a]All antigens were coated onto microtitre, as described in the methods, at a concentration of 5 μg/ml except for ssDNA which was used at 50 μg/ml.
[b]Expressed as fold increase over background (all reagents except test sample).

TABLE 2.8

RF Synergy with TRF in Reconstituting T-Depleted Cultures

Group	PFC/10^6 cells ± SE (*n*)
No Antigen	24.9 ± 9.1 (4)
Antigen[a]	30.6 ± 10.4 (4)
+ TRF high[b] Day 2	262.1 ± 25.5 (4)
+ TRF low[c] Day 2	59.5 ± 7.4 (4)
+ RFs 1-3 + 5[d] Day 0 and TRF low[c] Day 2	57.6 ± 11.9 (4)
	185.0 ± 7.5 (4)
+ RFs 1-3 + 5[e] Days 0-2	105.5 ± 11.0 (2)
+ RFs 1-3 + 5[d] Day 2	27.5 ± 0.9 (2)

[a]Sheep red blood cells added at culture initiation.
[b]Reconstituting dose of T-cell-replacing supernatant.
[c]One-tenth of reconstituting dose.
[d]One hundred nanograms of each of four RFs added to culture.
[e]Fifty nanograms of each of four RFs added per day.

TABLE 2.9

Lack of IL-4 Activity in RF4

2×10^5 B Cells[a] +	^{3}H-Thymidine Uptake (cpm/minute) ± SE
Medium (Control)	5402 ± 335
Con A 1 μg/ml	4182 ± 587
LPS 2.5 μg/ml	21692 ±1565
GαMμ 1 μg/ml	5665 ± 967
" 3 μg/ml	17500 ±2681
IL-4 1 ng/ml (20 U/ml)[b]	3593 ± 618
" 4 ng/ml (80 U/ml)	13712 ± 615
GαMμ 1 μg/ml + IL-4 1 ng/ml	6994 ± 489
" " + " 4 ng/ml	60787 ±8155
" " + RF4 .3 μg/ml(0hr)	3840 ± 304
" " + " " (2hr)	5407 ± 577
RF4 .3 μg/ml + IL-4 1 ng/ml	3522 ± 269
" " + " 4 ng/ml	12823 ±2178
GαMμ 1 μg/ml + RF supernatant[c]	3540 ± 386
RF supernatant[c]	3071 ± 237

[a]The cells remaining after T-depletion as outlined in the methods.
[b]One unit is defined by Genzyme Corp. as that amount of IL-4 which causes 50% maximal ^{3}H-thymidine uptake by 2×10^3 B cells in 100 μl cultures.
[c]100 μl of supernatant from RF4-reconstituted culture added per well.

TABLE 2.10

Reconstitution of Secondary T-Dependent Response[a]

Group	Indirect	Direct	IgG[b]
Not T-Depleted			
-Ag	164.0 ± 15.4 (4)[c]	64.3 ± 42.0 (4)	99.7
+Ag	675.2 ± 46.2 (4)	317.5 ± 57.8 (4)	357.7
+Ag + TRF	903.6 ± 14.7 (2)	569.4 ± 180.6 (2)	334.2
+Ag + RF 0hr	744.2 ± 143.9 (4)	308.3 ± 30.6 (4)	435.9
T-Depleted			
-Ag	61.3 ± 17.2 (4)	35.2 ± 22.2 (4)	26.1
+Ag	91.1 ± 18.3 (4)	36.0 ± 16.4 (4)	55.1
+Ag + TRF	403.1 ± 121.1 (4)	89.5 ± 10.4 (4)	313.6
+Ag + RF 0hr	311.7 ± 46.3 (6)	47.3 ± 28.6 (6)	264.4
+Ag + RF 48	185.2 ± 30.7 (2)	27.3 ± 18.2 (2)	157.9

[a]By analysis of variance (not including -Ag groups), p-values for effect of T cell depletion on IgM and IgG responses were 0.000 and 0.001, respectively. The presence of TRF or RF in the T-depleted groups affected the IgG response ($p=0.003$) but not IgM ($p=0.82$).
[b]Direct PFC subtracted from Indirect PFC.
[c]PFC/10^6 cells recovered ± SD (n).

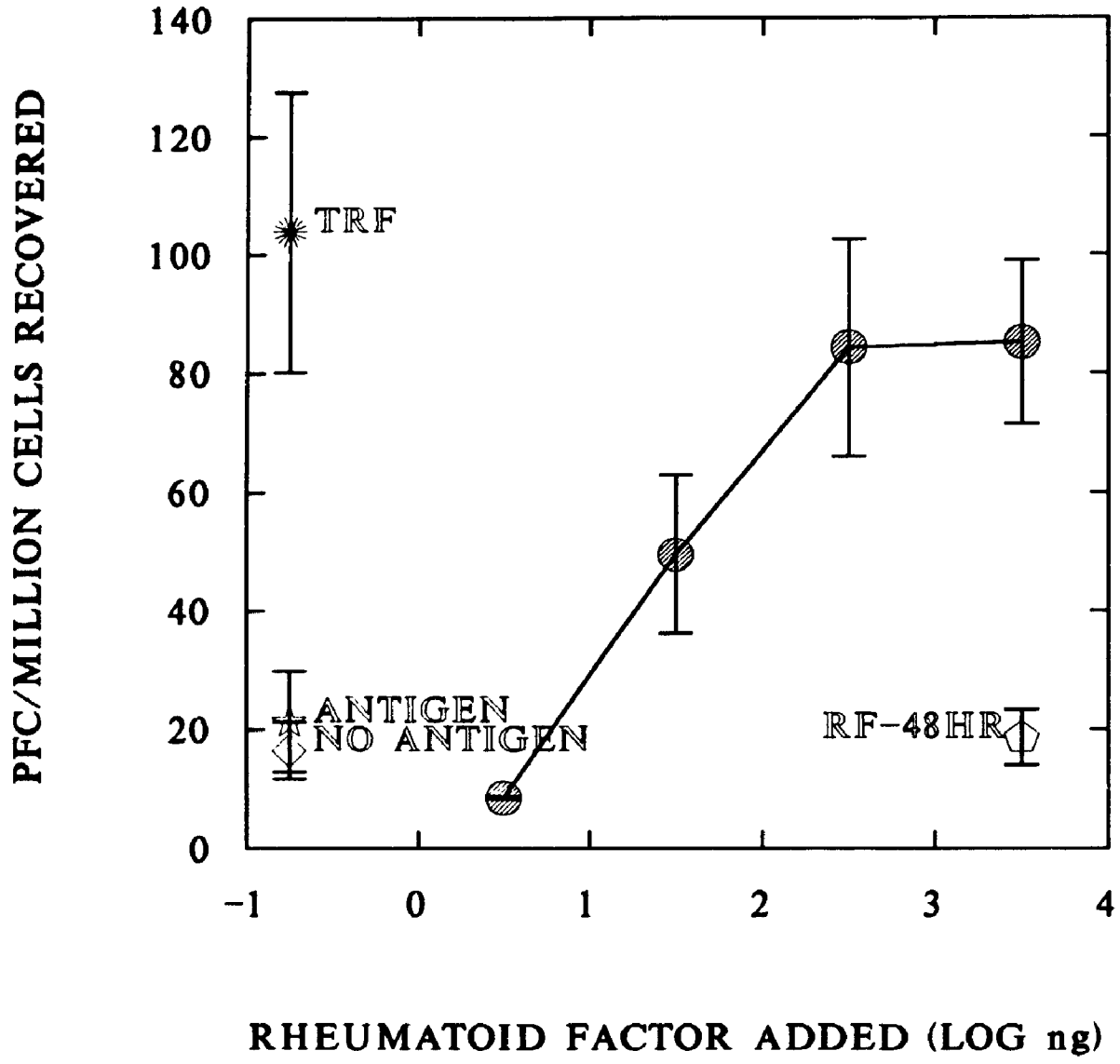

Figure 2.10. Dose-response curve of reconstitution of secondary anti-SRBC response by RF. T-depleted, SRBC-primed spleen cell cultures were either not stimulated (no antigen) or stimulated (antigen) with sheep erythrocytes. Antigen-stimulated cultures received RF3 at the indicated doses at either culture initiation (circles) or at 48 hours (hexagon), or a reconstituting dose of T-cell-derived lymphocyte-containing supernatants (TRF). Vertical bars indicate standard error of the mean.

4.4 DISCUSSION

This laboratory demonstrated Fc-dependent suppression of immune responses by IgG antibody, inactivation of lymphocytes by antigen-antibody complexes, and protection from Fc-dependent suppression by T cells (reviewed in Sinclair, 1983b; Sinclair and Panoskaltsis, 1987a; 1989). Also, a model for lymphocyte inactivation based on the crosslinking of antigen receptors (AgR) with Fc receptors (FcR) was proposed. These findings have been amply confirmed and extended by recent studies (Gadd *et al.*, 1985; Heyman and Pilstrom, 1988; Bruderer and Heusser, 1988), and negative signalling through FcγR has been correlated with alterations in the intracellular second messenger systems (O'Garra *et al.*, 1987; Wilson *et al.*, 1987) and the level of c-*myc* expression (Phillips and Parker, 1987).

The ability of goat anti-mouse IgG-Fc antibodies, added at culture initiation, to reconstitute a T-cell-dependent murine antibody response (Sinclair and Panoskaltsis, 1986a; 1986b; 1987a; 1987c) implicates end product antibody as an important regulatory component in the induction of immune responses. It was predicted (Sinclair and Panoskaltsis, 1986a; 1986b; 1987a; 1987c), and now the results of this thesis confirm, that RF, a naturally occurring Fc-binding agent, would exhibit a similar property. Most RF is of the non-suppressive IgM class and binds to the Fc portion of suppressive IgG particularly when the latter occurs in complexes (Coutelier and Van Snick, 1985). IgM RF is produced in large amounts in secondary immune responses in non-autoimmune animals (Van Snick and Coulie, 1983; Nemazee and Sato, 1983) and, since RF production follows the kinetics of IgG production (Stanley *et al.*, 1987), could also be generated in primary responses in low amounts at the time when high affinity IgG antibodies have also been demonstrated (Heyman and Pilstrom, 1988). RF may regulate primary and secondary immune responses by blocking negative Fc signals.

Since RF appears in both normal responses (Van Snick and Coulie, 1983; Coulie and Van Snick, 1985; Nemazee and Sato, 1982; 1983; Nemazee, 1985; Stanley *et al.*, 1987) and in autoimmune disease (Van Snick and Coulie, 1983; Coulie and Van Snick, 1985; Nemazee and Sato, 1982; 1983; Nemazee, 1985; Monestier *et al.*, 1986; Theofilopoulos *et al.*, 1983), a number of inducers have been suggested. In addition to induction by immune complexes containing IgG (Coulie and Van Snick, 1983; 1985; Nemazee, 1985;) and polyclonal activators (Izui *et al.*, 1979), RF may be produced following bacterial (Dresser and Popham, 1976) and/or viral (Vaughan, 1979) infection and could be the anti-idiotypic partners of antibodies against viral Fc-binding proteins (Mouritsen, 1986). Furthermore, RF may belong to a family of cross-reacting (Theofilopoulos *et al.*, 1983; Darwin *et al.*, 1986) or polyspecific (Ternynck and Avrameas, 1986; Rauch *et al.*, 1985; Cairns, E. *et al.*, 1986) autoantibodies or be induced by other antibodies expressing idiotypes which mimic Fc determinants (Holmdahl *et al.*, 1986).

RF may influence immune reactions (Levinson and Martin, 1988), but, the mechanisms are unclear. That RF may enhance the binding of antibody to antigen (Nemazee and Sato, 1982) is contradicted since RF has a greater affinity for bound IgG (Van Snick and Coulie, 1983) and is likely induced by bound IgG (Stanley *et al.*, 1987). RF may augment the binding of complement (Tesar and Schmidt, 1973; Clarkson and Mellow, 1981; Isturiz *et al.*, 1983), but RF can also interfere with C3b binding to IgG and, therefore, prevent IC deposition in the glomerulus (Bolton *et al.*, 1982). RF both enhances (Van Snick *et al.*, 1978; MacKinnon and Starkebaum, 1987) and inhibits (Devey and Hogben, 1987) IC clearance.

The experiments reported here confirm the previous prediction (Sinclair and Panoskaltsis, 1986a; 1986b; 1987a; 1987c) that RF is an important component in the regulation of immune responses. Not only does RF prevent Fc-dependent suppression of an immune response, RF reconstituted a T-cell-deprived system so

that a T-cell-dependent response could be generated. Therefore, the immune system has developed two mechanisms for dealing with negative Fc signals during the induction of an immune response: T cell functions (particularly, the production of IL-4 which has been shown to block negative Fc signalling (O'Garra *et al.*, 1987)) and RF activity. If RF is produced early following antigen stimulation (Stanley *et al.*, 1987), the resulting response may be less dependent on T-cell function.

The addition of small amounts of antigen-non-specific murine IgG, but not $F(ab')_2$ or IgM, will interfere with the ability of RF to reconstitute the T-cell-deficient system. This observation rules out the possibility that RF reconstituted the T-cell-deficient system through the generation of IC with adjuvant activity (Hobbs *et al.*, 1985) since ideal IC-forming conditions were available and polyclonal activation by stimulatory Fc subfragments cleaved from complexed IgG is dependent on both macrophages and T cells (Morgan and Weigle, 1983). This experiment also suggests that, for RF to have an immunoregulatory effect *in vivo*, it must act locally at the site of production by binding to specific endogenous IgG antibody attached to the stimulating antigen on the surface of the B cell. Other artifactual mechanisms (such as RF acting as a facilitating antibody for plaque formation due to carryover into the assay) are eliminated by the finding that RF, added later, did not elicit the antibody-forming cell response to antigen.

Suppression of antibody synthesis by an IgM anti-Fc antibody has been reported (Birdsall and Rossen, 1984); however, this system involved polyclonal activation by pokeweed mitogen rather than an antigen-specific mechanism in which end product feedback could operate. RF could have adversely affected a mechanism for Fc-dependent activation in the pokeweed model such as that described for the solicitation of B cell activating factors from Fc receptor-positive accessory cells (Goroff and Finkelman, 1988) due to stimulatory Fc subfragments (Morgan and Weigle, 1983). Observations based on mitogen activation are of

questionable interpretive value for understanding how lymphocytes are regulated, since mitogens use different, and most probably disturb, physiologic, regulatory pathways.

The elevated production of IgM RF in autoimmune disease (Boling *et al.*, 1987) may be a trigger, and not simply a mediator, of autoimmune disease. In normal responses, RF is predominantly of the non-suppressive IgM isotype (Van Snick and Coulie, 1983; Coulie and Van Snick, 1985; Nemazee and Sato, 1982; 1983; Nemazee, 1985). We [illegible] this as another mechanism, in addition to T-cell activity (Lees and Sinclair, 1975; Sinclair *et al.*, 1976; Hoffman and Kappler, 1978; O'Garra *et al.*, 1987), for overcoming negative Fc signals. In addition to an abnormally high level of RF, the ligand binding capacity of FcR (Laszlo and Dickler, 1988) may be reduced in autoimmunity. Since, in severe autoimmune disease, the RF is mainly of the IgG isotype (Theofilopoulos and Dixon, 1981), there may be other defects in negative Fc signalling, such as the inability of FcR to transmit these negative signals (Uher and Dickler, 1986b). Old autoimmune mice have a reduced number of $Fc\gamma R^+$ cells (Theofilopoulos and Dixon, 1981). Defects in reticuloendothelial FcR function are also found in RA (Fields *et al.*, 1983), suggesting that abnormalities in FcR are a general phenomenon in this disease, Since the carbohydrate moiety of the Fc is required for negative signalling (Heyman *et al.*, 1985), the abnormal glycosylation of IgG, noted in rheumatoid arthritis (Parekh *et al.*, 1985; Rook, 1988), would lead to defective feedback.

Since "internal image" anti-idiotypic antibodies for RF displace RF from immune complexes (Nelson *et al.*, 1985; Fong *et al.*, 1986), failure to produce these anti-idiotypic antibodies could result in abnormal B-cell activation since RF would be free to block Fc-mediated suppression. The image bearing IgG anti-idiotypes for RF could reduce RF production (Koopman *et al.*, 1983; Abe *et al.*, 1984) by crosslinking, in either direction, AgR and FcR on RF-specific B cells. On the other

hand, IgM image bearing anti-idiotypes would bind only at the $F(ab')_2$ end and could prevent Fc feedback on any B cell or allow only inhibition of differentiation but not proliferation (Uher and Dickıer, 1986a), thus establishing a situation conducive for intense RF production once the negative Fc signal dissipates.

There is some indication in the present results that the effectiveness in reconstituting the T-cell-dependent response may relate to the IgG isotype recognized by the particular RF; however, the results could also be interpreted in terms of a specific Fc signalling region shared by all IgG isotypes and recognized by only some of the RFs used.

The T-cell requirement for production of IgM RF (Coulie and Van Snick, 1985; Nemazee and Sato, 1982; 1983; Nemazee, 1985) could result from the focussing of immunosuppressive IgG-Ag complexes on the surface of RF-specific B cells. The abnormal TD response in MRL/lpr mice, much of which is of the IgG_{2a} isotype (Park *et al.*, 1983), could represent an attempt to down regulate the abnormal response in these mice; down regulation may be frustrated because of the incompetency of the $Fc\gamma R$. Further regulatory defects in RA such as decreased T cell-mediated inhibitory activity (Olsen and Jasin, 1987) may be due primarily to the abnormal production of RFs which prevent the Fc-dependent, IC-induced release of immunoglobulin binding factors, the immunosuppressive soluble form of $Fc\gamma R$, from inhibitory T cells (Fridman *et al.*, 1981).

The synergism observed between early Fc signal blockade and later TRF activity finds an interesting parallel with a recent paper (Hobbs *et al.*, 1987) which reported reconstitution of a T-cell-depleted lymphocyte population with an Fc fragment, p23, and TRF. The Fc fragment, which had been previously shown to interfere with tolerance induction (Walker *et al.*, 1984), may block negative Fc signals at the B-cell level by binding the FcR and thus prevent AgR-FcR crosslinking. Fc subfragments cannot crosslink FcR to suppress differentiation

(Uher and Dickler, 1986a) and, moreover, they may prevent FcR aggregation by other agents, such as nonspecific IC, which could reduce the degree of responsiveness. That RF, multiply administered to cultures markedly deficient in T cells, can reconstitute even without the further addition of TRF suggests that the physiologic action of the late-acting TRF is also linked to the attenuation of negative Fc signals.

Aside from preventing the crosslinking of FcγR and AgR, IgM RF could also favor the crosslinking of FcμR (Mathur *et al.*, 1988; Pichler and Broder, 1978) with AgR on the surface of B cells. This may result in the delivery of an independent positive signal or prevent the negative signal resulting from the interaction between FcγR and AgR through an Ag-IgG antibody bridge (Sinclair and Chan, 1971).

Thus, an antigen-non-specific, but antigen-directed, mechanism for regulation of B-cell induction can be blocked by the addition of RF. RF not only interferes with Fc-dependent antibody-mediated immunosuppression, but also, RF reconstitutes a T-dependent antibody response by T-cell-depleted cultures. These findings indicate that immune complexes, formed early in immune responses, down regulate by crosslinking AgR with FcR on the B-cell surface and that RF can interfere with this crosslinking, thus replacing T-cell help. This implies that the rate-limiting, if not the only, function of "helper" T cells is the abrogation of negative Fc signalling. Increased production of RF may deregulate a negative end product feedback mechanism needed to control autoimmunity (Sinclair and Chan, 1971).

CHAPTER 3 LACK OF HIGH AVIDITY ANTI-ssDNA Ab IN AUTOIMMUNE MICE

3.1 INTRODUCTION

The serological and pathological similarities to human systemic lupus erythematosus (SLE) makes the New Zealand (NZ) mouse model useful in providing insights into the etiology of autoimmunity especially since it allows one to study these mice at the "pre-autoimmune" stage. Of its numerous immunologic features, the most salient is the high titers of anti-DNA/RNA/histone antibodies which result in immune complex deposition and complement activation associated with glomerulonephropathy (Theofilopoulos and Dixon, 1981; Klinman, 1989; Tan, 1989; Tan *et al.*, 1988; Stollar and Schwartz, 1986; Bach *et al.*, 1986; Datta and Gavalchin, 1986; Schmiedeke *et al.*, 1989; Fisher *et al.*, 1988, to name a few).

Although there is still doubt about the exact specificity of antibodies which cause the greatest tissue damage in SLE and its New Zealand mouse equivalent (see above references), histologic and serologic investigations have given us a fairly detailed understanding of the pathology of the disease in which glomerular deposits of IgG, C3 and DNA accumumlate on the glomerular basement membrane of the kidney and result in the fatal IC glomerulonephritis (reviewed in Theofilopoulos and Dixon, 1981). However, the etiology of lupus is still unclear. Many aberrations have been reported including hypo- and hyper-activity in practically every compartment in the immune system. Among the many examples are increased T helper cell activity in NZB mice (Laskin *et al.*, 1986; Cowdery and Pitts, 1988), B/W mice (Ando *et al.*, 1987; Wofsy and Seaman, 1987; Carteron *et al.*, 1989), decreased T suppressor cell activity in B/W mice (Steward and Hay, 1976; Sekigawa *et al.*, 1987), defective lymphokine production in MRL/*lpr* (Santoro *et al.*, 1987) and B/W mice (Jacob *et al.*, 1987), overexpression of a minor $CD4^-CD8^-$ T helper cell subset (Datta *et al.*, 1987; Sainis and Datta, 1988; Theofilopoulos *et al.*, 1989),

hypersensitivity of autoreactive B/W B cells to helper signals (Herron *et al.*, 1988a; 1988b; Umland *et al.*, 1989), the production of a specific immunogenic molecule (Pancer *et al.*, 1980; Atkinson *et al.*, 1985) and defective B cell tolerance (Goldings *et al.*, 1980; Goldings, 1988). Still others have found no decreased T suppressor cell activity (DeMoor *et al.*, 1985) and that there must be an intrinsic defect in the B cell compartment such as inability to recognize suppressive message (Primi *et al.*, 1978), early B cell maturity and hyperactivity (Theofilopoulos *et al.*, 1980) and the presence of polyclonal activators (Jyonouchi *et al.*, 1985; Prud'homme *et al.*, 1983; Hirano *et al.*, 1987).

The key to understanding the etiology of systemic lupus, as well as many other autoimmune diseases, is a knowledge of how the immune (and autoimmune) response is normally regulated and what goes wrong with this regulation to lead to clinical autoimmunity. One must identify the changes in the *capacity* of the immune system to generate autoantibodies of various avidities as well as changes in both the amount and avidity of autoantibodies which are actually produced in autoimmunity. Although it is generally agreed that higher amounts of anti-DNA antibodies are associated with SLE symptoms (Tron and Bach, 1977) and higher avidity cationic IgG antibodies tend to localize in the kidneys (Winfield *et al.*, 1977), there is no correlation between the amount and the avidity of the antibodies produced in SLE (Asano and Nakamoto, 1978). Attempts to delineate the nature and the quality of the anti-DNA antibodies produced have divided the literature into two "camps" which basically do not share much in terms of common ground because of differing methodologies (some of which measure only high avidity antibodies) and the differing sources of antibody studied. Some investigators have reported correlations of high avidity antibodies with severe SLE (Leon *et al.*, 1977; Pearson and Lightfoot, 1981) and lower avidity associated with milder forms of the disease (Smeenk *et al.*, 1982a; 1982b). Others have found the contrasting presence of low avidity anti-DNA

antibodies in the serum and high avidity in glomerular eluates (Winfield *et al.*, 1977) while low avidity antibodies have been found to correlate with severity of disease (Riley *et al.*, 1979) due to inefficient IC clearance caused by poor lattice formation (Morgan and Steward, 1976). Susceptibility to disease is seen in mice either with a predisposition to produce low avidity antibodies (Steward *et al.*, 1975) or inbred for low avidity antibody production (Devey *et al.*, 1984). Furthermore, since sequestration of autoantibodies in immune complexes undoubtedly takes place (Harbeck *et al.*, 1973; Cronin *et al.*, 1986), experimental approaches must address autoantibody *production*, unaffected by sequestration so that what is measured reflects what is produced.

In this chapter, the spontaneous production of anti-ssDNA antibody synthesizing, plaque forming cells (PFC) *in vitro* (Sawada *et al.*, 1977; Roder *et al.*, 1978a; Sawada and Talal, 1979; Fish and Ziff, 1982) was studied for three reasons. First, in view of the literature on this subject, it was appropriate to study the avidity of anti-ssDNA antibody as it is *produced*, not as it remains after complexing with DNA. Second, it was necessary to set up a *comparison* between normal and autoimmune-prone mice for the production of antibody with specificity for at least one of the target antigens involved in an autoimmune disease. If the autoantibody occurred only in clinical autoimmunity, no comparison of amounts or avidity of antibody produced, between normal and autoimmune-prone mice, could be made. Third, the major emphasis was on the comparative *capacity* of the immune system of normal and autoimmune mice to produce antibody with specificity for a self-antigen. For this purpose, the spontaneous production of anti-DNA antibody by spleen cells in culture (Roder *et al.*, 1978a) is ideal, since it represents the functioning of lymphocytes after release from control processes which operate *in vivo*.

By approaching autoreactivity in this fashion, it was possible to study the level at which the lymphoid system normally prevents tissue damaging anti-self

immune responses. It is already known that IgM anti-ssDNA antibodies are produced in the absence of lupus (Sawada *et al.*, 1977), while the switch to IgG anti-ssDNA antibodies is much more closely associated with the disease state (Talal *et al.*, 1976; Steward and Hay, 1976; Papoian *et al.*, 1977). It is this pre-switch phase of B cell activation in normal and autoimmune-prone animals which is the focus of this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Male and female CBA, NZB, NZW and NZBxNZW F_1 mice (6 weeks of age) were purchased from Jackson Laboratories, Bar Harbor, Maine. DBA/2 were obtained from Charles River Canada Inc., St. Constant, Quebec. Athymic, homozygous nu/nu (nude) mice of the RNC line (Croy and Osoba, 1974) were purchased from the lab of Dr. B. Bloomfield, University of Guelph (Guelph, Ontario). All mice were housed in periodically changed, autoclaved micro-isolater units with filters (Lab Products, Inc., Maywood, New Jersey), and kept on an oral diet of sterile mouse food pellets and water.

3.2.2 Preparation of Serum from Mice

Mice were bled by cardiac puncture and the blood allowed to clot at room temperature. The clot was then removed, the serum was centrifuged twice for 30 minutes at 2,000 rpm at 4^{o}C and stored at -20^{o}C.

3.2.3 Cell Culture

All media and supplements used are described in chapter 2.2.2. Spleen cells from individual mice were cultured and assayed separately; spleens from mice in various groups were not pooled. One-milliliter Mishell-Dutton cultures (1967) of unfractionated splenocytes at 10^7 cells/ml of complete minimal essential medium were established in quadruplicate in 24-well flat-bottom tissue culture plates (Falcon 3047) without antigen, in order to generate spontaneous anti-ssDNA antibody production. Cultures were kept at a 10% CO_2 atmosphere on a rocking platform at 37^{o}C and fed with 50 μl nutritive cocktail each day for 5 days.

3.2.4 Coupling of SRBC with ssDNA

To determine the number of anti-ssDNA PFC, sheep erythrocytes (Woodlyn Laboratories Ltd., Guelph, Ontario) were coupled with ssDNA by the chromium chloride method as described (Clarke *et al.*, 1972). Calf thymus DNA Type 1

(Sigma Chemical Co., St. Louis, Missouri), shown to be relatively pure by the ratio of absorbance at 260nm/280nm (> 1.8), was made single-stranded by boiling for 15 minutes then rapidly freezing in an ice-salt bath. Denaturation of DNA was confirmed by its hyperchromicity (increase in absorbance at 260nm). To a series of 8 tubes, 1 ml of ssDNA at 500 μg/ml PBS was mixed with 0.1 ml of 0.02% $CrCl_3$ (J. T. Baker Chemical Co.) in saline. After 3 to 4 minutes, 0.2 ml of 50% SRBC (in saline) were added and each tube shaken lightly by hand for 10 minutes. Upon addition of 0.8 ml saline (0.85% NaCl), tubes were shaken by hand for 2 minutes and the reaction stopped by the addition of 5 ml cold PBS. Coupled SRBC were centrifuged at 2000 rpm for 5 minutes and subsequently washed 3 times in PBS and a final wash in BSS. Cells were suspended at 1:15 in BSS (0.5 ml pelleted cells + 7 ml BSS) for use as targets in the plaque assay and at 1:100 (0.05 ml pelleted cells + 5 ml BSS) for the hemagglutination test.

Hemagglutination was used to check for coupling of SRBC with ssDNA. The anti-DNA antibody-containing standard serum used was that of a lupus patient and obtained as a kind gift of Dr. D. A. Bell (University Hospital, London, Ont.). Serial 2-fold dilutions of normal horse serum starting at 1:2 (FLOW Labs) or anti-DNA serum beginning at 1:80 were made in duplicate in 96-well V-bottom plates (Dynatech) containing 50 μl PBS per well. Fifty microlitres of ssDNA-coupled or non-coupled SRBC were added to one row of each serum set. Wells were left undisturbed for 2 hours and hemagglutination noted. SRBC were considered adequately coupled if hemagglutination was noted up to at least the 1:320 dilution of anti-DNA standard serum and none (or only at 1:2) with normal horse serum. All wells which were given non-coupled SRBC exhibited no agglutination and, thus, the suitability of the ssDNA-coupled SRBCs for use as anti-ssDNA targets for hemolytic assays was confirmed.

3.2.5 Plaque Assay

Quadruplicate cultures from the same spleen were pooled for the assay. Hemolytic plaque forming cell assays were performed using a modification of the method by Jerne *et al.* (1974) as described previously (Bell *et al.*, 1973). Briefly, 500 μl of 0.5% Sea-Plaque agarose (Mandel) was mixed with 50 μl of target cells (either ssDNA-coupled or not coupled SRBCs) in small fermentation-type glass tubes set up in a 42°C water bath. Spleen cells taken from cultures were washed twice and resuspended in appropriate volumes of BSS (usually 3-fold the culture volume). One hundred microlitres of the cell suspension was added to the agarose-target cell mix, and the mixture in a total volume of 650 μl was poured onto frosted-end slides which had been precoated with 0.1% agarose. Facilitating rabbit anti-mouse Ig sera and Hemo-Lo guinea pig complement were purchased from Cedarlane, and were used as described in chapter 2.2.12. The number of anti-SRBC PFC (against uncoupled SRBC targets) was subtracted from that using ssDNA-coupled SRBC in order to obtain the net number of anti-ssDNA specific PFC.

3.2.6 Plaque Inhibition Assay

Increasing amounts of free ssDNA were incorporated into the agar used in the assay (in 0.5% agarose at concentrations starting from 0.005 μg to 5 μg DNA per slide which contained a total volume of 650 μl) to inhibit the formation of anti-ssDNA plaques. The amount of free ssDNA needed to cause 50% inhibition was used as a measure of the relative avidity of antibodies formed in plaques (determined from a plot of % inhibition versus the amount of DNA added on a log scale). High avidity antibodies in plaques are more easily inhibited by free antigen (i.e., less free ssDNA is needed to cause inhibition) than those of low avidity (i.e., more ssDNA needed). The rationale for this plaque inhibition assay has been described (Andersson, 1970; DeLisi and Goldstein, 1974). Briefly, as antibodies diffuse through the agarose towards the SRBC targets they must pass through a

"sea" of antigen (DNA). If this antigen is recognized with high avidity, there is a propensity for the antibody to complex with the free antigen rather than to continue diffusing towards that coupled to the target. The higher the avidity for the antigen, the less free antigen is needed to inhibit plaque formation. Plaque size can also be affected by avidity but since spleen cells from all strains of mice produced heterogeneous sizes of anti-ssDNA plaques (i.e. all strains produced large and small plaques), plaque size was not taken into consideration.

3.2.7 DNAse Digestion of Sera and Supernatants

DNAse 1 has been shown to release anti-DNA antibody bound to DNA (Deicher *et al.*, 1959). In order to free any anti-DNA antibody which could be hidden in DNA-anti-DNA complexes, all sera and supernatants were digested with DNAse 1 prior to testing by ELISA according to the method described by Cronin *et al.*, 1986.

DNAse 1 (Sigma, approx. 2200 units/mg solid), from bovine pancreas, was ved at 1 mg/ml borate-buffered saline (0.05 M borate-BDH, 0.1 M NaCl, pH 8.0) containing 0.04 M $MgCl_2$ (BDH). Twenty microlitres of heat-inactivated serum (56°C, 30 min) were diluted 2-fold with ELISA washing buffer, mixed with 20 μl DNAse 1 and incubated for 2 hours at room temperature. The reaction was stopped with 35 μl 0.08 M Na_2EDTA (pH 8.0) and the serum diluted appropriately (usually to a final dilution of 1:100). Supernatants were treated in the same way using 100 μl supernatant, 40 μl DNAse and 60 μl EDTA, and diluted to a final dilution of 1:8. DNAse digestion of sera and supernatants from all strains of mice consistently resulted in higher binding to ssDNA, as determined by ELISA (data not shown). Total Ig determinations also were correspondingly higher. The results obtained when EDTA was added before DNAse (and therefore inhibited its action) did not differ from non-digested aliquots of the same test sample.

3.2.8 Antibody Determinations

The enzyme-linked immunoassays employed the biotin-streptavidin procedure (Voller *et al.*, 1980; Hagen and Strejan, 1987) using 100 μl volumes in microtiter wells (Falcon 3040). For anti-ssDNA antibody determinations, plates were coated overnight at 4°C with ssDNA (50 μg/ml PBS), blocked for 2 hours at room temperature with 1% gelatin (Fisher) in PBS, incubated with DNAse 1-digested sera or culture supernatant (1 hr, 37°C) followed by biotin-labelled anti-immunoglobulin (Jackson ImmunoResearch, 1:5000 dilution, 2 hr at room temperature), then with phosphatase conjugated streptavidin (Jackson ImmunoResearch, 1 hr 37°C), developed for 20 min with p-nitrophenyl phosphate disodium (1 mg/ml diethanolamine buffer; Sigma/BDH Chemicals as per section 2.2.15) and fixed with 3N NaOH, with washes between each step (0.01 M PBS, 0.05% Tween 20, 0.2% gelatin, and 0.02% Na-azide). Absorbance was read at 405 nm on an ELISA plate reader (Flow). Determination of murine rheumatoid factor (RF) followed the same procedure as described in section 2.2.15. Each sample was examined as a serial dilution and compared to standard curves of mouse IgG or IgM (Sigma) dilutions run on the same plate. Total antibody levels and IgG or IgM standard curves were evaluated on goat $F(ab')_2$ anti-mouse $F(ab')_2$ and goat-anti-mouse IgM (μ chain) coated at 5 μg/ml PBS.

3.2.9 Inhibition ELISAs

Increasing amounts of ssDNA (prepared as in section 3.2.4) were added to DNAse 1 digested supernatant or serum in order to obtain inhibition curves as determined by the decrease of the absorbance reading in the ELISA. Single-stranded DNA at concentrations of 0.002 μg to 20 μg per ml washing buffer were added to equal volumes of the sample to be tested in a series of borosilicate glass tubes (usually 500 μl of each for a total volume of 1 ml). Tubes were incubated for 1 hour at 37°C to reach equilibrium and then samples were added to the ELISA

plates and developed as described to determine the level of binding to DNA and total Ig levels remaining.

3.2.10 Calculation of Anti-DNA Antibody Avidities (Dissociation Constant - K_D)

The dissociation constant of the antigen-antibody equilibrium was calculated by the Klotz equation as described by Friguet *et al.*, 1985:

$$K_D = [(A_0\text{-}A/A_0) - 1] \text{ X } a_0$$

where K_D is the dissociation constant, $moles^{-1}$

A_0 is the absorbance (O.D.) reading in the absence of inhibitory DNA (i.e., washing buffer only)

A is the absorbance in the presence of the inhibitory DNA,and

a_0 is the molar concentration of inhibitory DNA (molecular weight approximately 10^6).

3.2.11 Statistical Analysis

The significance of differences between groups was determined by analysis of variance and multiple comparison tests (Zar, 1984). Multivariate analyses of variance and multiple regression coefficients (Wilkinson, 1988) were employed. The graphic representation was created using SYGRAPH (Systat, Inc, Evanston, Illinois).

3.3 RESULTS

3.3.1 Development and Specificity of the Anti-ssDNA Plaque Assay

As shown in Table 3.1, ssDNA was effectively coupled onto the surface of SRBCs by the chromium chloride method as demonstrated by hemagglutination tests. The anti-DNA antibody-containing standard serum of a lupus patient was able to agglutinate ssDNA-coupled SRBC (titre of 2560) and not non-coupled SRBC. Normal horse and fetal calf sera from two different sources had low titres for ssDNA-SRBC. A high concentration of an irrelevant high MW protein (bovine serum albumin) did not agglutinate either ssDNA-coupled or non-coupled SRBC and, hence, the hemagglutination seen is antigen specific and confirms the ssDNA-coupled nature of the SRBCs which were used for the plaque assays.

Development of the anti-ssDNA plaque assay entailed the determination of the conditions under which plaques could be most clearly defined since other laboratories report some technical difficulties with this assay (personal communication with lab personnel). Table 3.2 shows that when facilitating serum (rabbit-anti-mouse Ig) was added as a separate incubation before the incubation in guinea pig complement (condition A) as opposed to being added together with complement (condition B), anti-DNA plaques were not only more numerous but also easier to read. When facilitating serum was incorporated into the agar (condition C), no plaques could be read and, in fact, complete lysis of SRBCs was evident. A few slides were picked at random in all experiments and examined under microscope to confirm the presence of one lymphocyte at the center of a plaque. Use of SRBC-absorbed guinea pig complement (Hemo-Lo C, Gibco) gave higher counts and had a profound effect on the clarity of the plaques. Primary anti-SRBC plaques from SRBC-stimulated cultures were, in fact, inhibited by those conditions which were conducive for good anti-ssDNA plaques (especially addition of facilitating serum). This is in agreement with reported plaque assay protocols

(Henry, 1980). Plaques seen are not a consequence of artifact in the assay since spleen cells taken fresh from the animal gave an extremely low response as compared to that of cells after 5 days of culture, the time of the peak spontaneous anti-ssDNA response (Roder *et al.*, 1978a).

The specificity of the anti-ssDNA plaques was demonstrated by inhibiting plaque formation using increasing concentrations of free ssDNA incorporated into the agarose (Figure 3.1). Free ssDNA did not inhibit either background SRBC or primary anti-SRBC plaque production, and therefore, the specificity of the anti-DNA plaques was confirmed. Fine specificity of plaque formation is shown in Figure 3.2. Incorporation of native DNA into the agarose could only inhibit anti-ssDNA plaques at high concentrations and even then at only a fraction of that obtained using ssDNA. This probably indicates that some plaques are against epitopes common to single- and double-stranded DNA and also that native DNA preparations contain single-stranded regions as has been reported (Locker *et al.*, 1977).

3.3.2 Decreased Avidity of Spontaneous anti-ssDNA Autoantibody from an Autoimmune Strain of Mice

It can be seen in Table 3.3 that significantly more free ssDNA is needed to cause 50% inhibition of plaque production of spleen cells from the NZB/W F_1 autoimmune strain of mouse in comparison with non-autoimmune strains (normal CBA and athymic nu/nu mice). Because more free antigen is needed to inhibit plaque formation by antibodies of low avidity as opposed to higher avidity plaques, the results of Table 3.3 indicate that strains from an autoimmune genetic background have significantly lower avidity anti-ssDNA antibodies than normal or non-autoimmune mice. This result is not due to differences in number of plaque-forming cells since all groups of mice were able to generate similar numbers of plaques.

3.3.3 Avidity changes with age in normal and autoimmune strains of mice

A three-way correlation is depicted in Figure 3.3 between the avidity of anti-ssDNA PFC (expressed as the quantity of free ssDNA required to give 50% inhibition of plaque numbers), the number of anti-ssDNA PFC, and the age of the mice. The main observation is that the avidity of anti-ssDNA PFC is *higher* in *young non-autoimmune-prone* mice than in autoimmune prone mice. This elevated avidity, seen in the young non-autoimmune-prone mice, decreases slightly with age (Tables 3.4 and 3.5). Even at 20 weeks of age, the avidity in normal mice is higher (50% inhibition point is lower in the XZ panel of Figure 3.3) than in the autoimmune-prone mice. Therefore, while lymphocytes from non-autoimmune-prone mice can generate antibody forming cells with high avidity for autoantigen, the autoimmune strains cannot do so in the age range studied (6-48 weeks).

There is a significant correlation between number of PFC and avidity (Figure 3.4). However, the coefficient of determination was only 0.09 indicating that a mere 9% of the variation in avidity can be explained on the basis of a variation in number of PFC. Since there was no change in the number of anti-ssDNA PFC in non-autoimmune mice with age (Table 3.6), the change in avidity is the dominant event. The change in avidity also dominated over the polyclonal spontaneous background antibody production (Table 3.8) and cell viability in culture (Table 3.7).

Table 3.4 gives the mean dose of soluble ssDNA needed to cause 50% inhibition of anti-ssDNA PFC in non-autoimmune and in two autoimmune-prone strains, and their F_1 hybrid, grouped according to age. Anti-ssDNA PFC were inhibited by significantly lower concentrations of soluble ssDNA in the non-autoimmune mice than in the autoimmune strains. This difference was significant at a p-value < 0.001, using analysis of variance and multiple comparison tests. There was no difference between the sexes within the autoimmune and normal strains

(Table 3.5) except for the low avidity antibody produced by the female normal mice in the middle age group. However, the small sample size of this group, and larger standard error compared to the other normal groups, limits its significance.

A multivariate analysis of variance (Zar, 1984; Wilkinson, 1988) on the effect of mouse strain and age on the number and avidity of PFC demonstrated a significant impact of strain derivation but no effect of age on avidity (Wilks' lambda F-statistic, and Pillai and Hotelling-Lawley trace F-statistics all evaluating to a p-value of 0.001 for strain derivation and to 0.1 for age). In a univariate analysis of variance, the interaction between mouse strains and age groups was significant ($p=0.035$) with respect to avidity (indicating that age affected avidity differently, depending on the autoimmune status of the mice).

3.3.4 Effect of age and mouse strain on the number of spontaneous anti-ssDNA PFC

Figure 3.3 and Table 3.6 show the effect of age and mouse strain on the number of spontaneous anti-ssDNA PFC produced in culture. No significant differences were evident, except with cells from old NZW mice which showed a decreased PFC production compared to young NZW mice. Overall, mouse strain did not affect the number of anti-ssDNA PFC/culture ($p=0.255$) except with cells from two groups: 1) the youngest NZB mice which generated fewer PFC and, 2) the oldest NZB/NZW F_1 which produced higher plaque numbers, when compared to the other strains in their respective age groups. The same conclusions were drawn whether PFC counts were expressed as PFC/culture or as PFC/million viable cells recovered, since cell viability was not affected by the age or strain of mouse (Table 3.7). Although the spontaneous or background production of anti-SRBC plaques (using non-coupled SRBC) was generally higher in the autoimmune strains (Table 3.8), the differences were not enough to mask potential differences in anti-ssDNA plaque counts, or be the cause of the reduced ability to inhibit anti-ssDNA plaques

in cells from the autoimmune strains. Spontaneous and primary anti-SRBC plaques were not inhibited by free ssDNA (data not shown).

3.3.5 Antibody production in cultures and in vivo

Determinations of secreted anti-ssDNA antibody and RF were made for two reasons. First, since the results from the plaque-inhibition studies could conceivably be interpreted as being due to differing amounts, rather than differing avidities, of anti-ssDNA antibody produced, an estimate of the amounts of antibody secreted is informative. Second, although the strains of mice used were obtained from a recognized source, the presence of autoimmune characteristics should be confirmed and reported. The accuracy of antibody determinations was confirmed by comparison of the levels of IgM in the RF preparations used (in chapters 2 and 4 of this thesis) as determined by our ELISA method with those determined by Dr. P. Coulie who kindly donated them. Table 3.9 shows good agreement (some excellent) between determinations from the two labs and, thus, the ELISA employed can be considered adequately accurate for measurement of antibody levels since it is mainly the *comparison* between non-autoimmune and autoimmune mice which is of interest.

The *in vitro* production of IgM anti-ssDNA antibody by lymphocytes from normal mice equalled, and in many cases exceeded, that obtained with cells from autoimmune-prone mice (Table 3.10). This suggests that there may, indeed, be a difference in the amount of IgM anti-ssDNA antibody formed per cell, in culture, between normal and autoimmune-prone mice (since the PFC counts did not differ), but the direction of the difference is opposite to that needed to explain the results of the plaque-inhibition studies as an artefact of amount of antibody produced per PFC. The lower avidity of anti-ssDNA antibody formed by cultured cells from autoimmune mouse strains may, in fact, be underestimated. There were no significant differences in IgM anti-ssDNA antibody levels between age groups

except for old B/W mice whose lower IgM levels were counterbalanced by the switch to IgG-anti-ssDNA characteristic of this strain (data not shown).

The level of secreted IgG anti-ssDNA antibody was higher in the F_1 mice, both *in vitro* and *in vivo*, than in all the other strains (Table 3.10). The *in vitro* IgG anti-ssDNA production represented a minority of the anti-ssDNA antibody produced by all strains, while the *in vivo* shift to IgG anti-ssDNA antibody was apparent in the NZB and F_1 mice. The production of RF in the F_1 and NZW mice is not elevated while the NZB mice demonstrate an elevated capacity to produce RF, both *in vitro* and *in vivo* (Table 3.11). There were no significant differences or trends among age groups except for old (26-48 week) NZB mice whose RF levels increased two to four fold across the board (data not shown). Therefore, the immunologic stigmata of autoimmunity are demonstrable in these mice.

The amount of free ssDNA needed to cause 50% inhibition of plaques correlated ($r^2 = 0.04$, $p = 0.05$) with the anti-ssDNA antibody level in the culture supernatant (Figure 3.5) so that ease of plaque inhibition showed a significant but limited relation (4%) to the quantity of anti-DNA antibody in the culture supernatant. The level of IgM-anti-DNA antibody produced in culture strongly paralleled the total IgM level (Figure 3.6) while RF levels against all IgG specificities did not (Figure 3.7). Hence, this *in vitro* system does not allow for the expansion of all B cells but does preferentially so for at least the anti-ssDNA antibody-producing cells. Furthermore, there was a strong correlation between numbers of anti-ssDNA plaques and IgM-anti-ssDNA antibody supernatant levels (Figure 3.8) and not with IgG-anti-ssDNA (Figure 3.9) which means that the plaque assay was most suited for the measurement of IgM plaques.

3.3.6 Determination of K_Ds of Anti-ssDNA Antibodies by Inhibition ELISA

Dissociation constants (K_D) of anti-ssDNA antibodies from culture

supernatants and sera (from the same mice) were determined by inhibition ELISA as described by Friguet *et al.* (1985). Figure 3.10 shows that the relative measure of anti-ssDNA antibody avidity as determined by plaque inhibition does not correlate with K_D measurements by ELISA (the higher the K_D, the lower the avidity) using culture supernatants. Also, no correlation was seen with serum anti-DNA antibodies (Figure 3.11). A plot of dissociation constant versus the concentration of anti-ssDNA antibody showed no correlation (Figure 3.12) and, therefore, the K_D measured was not a function of antibody concentration. There were no strain differences with respect to any of the correlations. In interpreting the discrepancy between the two methods for measuring avidity, the use of plaque assays not only avoids the problem of antigen and antibody sequestration which takes place *in vitro* and *in vivo*, it also gives the "before picture" of the antibody population produced preceding the uptake and breakdown of immune complexes (most likely those containing high avidity antibodies) by macrophages. The number of anti-ssDNA PFC produced did not correlate with K_D (Figure 3.13) and, hence, a gamut of avidities is measured in the plaque assay and not only high avidity. Therefore, measurement of the relative avidities of antibodies which the immune system is capable of producing is viable and more reproducible using the plaquing technique.

TABLE 3.1

Hemagglutination Tests of ssDNA-Coupled SRBC

Group	Titre in presence of SRBC	Titre in presence of ssDNA-SRBC
Horse Serum (Flow)	0	2 (±)[a]
Horse Serum (Gibco)	0	32 (±)
Fetal Calf Serum (Flow)	0	8 (±)
Fetal Calf Serum (Gibco)	0	32 (±)
Anti-DNA Standard	0	2560 (±)
Bovine Serum Albumin (50mg/ml)	0	0

[a](±) = Marginal agglutination at that titre.
The same results were obtained when done before and after plaque assay.

TABLE 3.2

Determination of Conditions for Anti-ssDNA Plaque Assay

Group	Guinea Pig Complement			
Facilitating Serum	Not SRBC-Absorbed		SRBC-Absorbed (Hemo-Lo)	
R-anti-mouse Ig	α-DNA	α-SRBC	α-DNA	α-SRBC
5-Day cultured cells[a] +				
No Antigen				
A-separate incubation	580±231(6)[a]	48±23(6)	920±461(6)	60±47(6)
B-added with c'	207±119(8)	46±14(8)	325±16(4)	130±100(4)
C-in agarose	complete lysis			
D-none	168± 42(8)	181±51(8)	148±76(6)	135±49(6)
Antigen (SRBC)				
A	385±65(2)	145± 5(2)	755±237(4)	123±45(4)
B	320±30(4)	257±43(4)	405± 5(2)	290±10(2)
C	complete lysis			
D	320±63(4)	330±46(4)	373±208(4)	575±223(4)
Fresh cells[c] +				
No antigen				
A	105±45(4)	12± 8(4)	65±15(2)	35±25(2)
B	25± 8(4)	35±21(4)	30±10(2)	5± 5(2)
D	5± 5(4)	32±19(4)	15± 5(2)	10± 0(2)

There were no plaques in the absence of complement.
[a]Cell viability between 22-26%.
[b]Expressed as PFC/culture ± Standard Deviation (n).
[c]Cells made up to concentrations comparable to other groups.

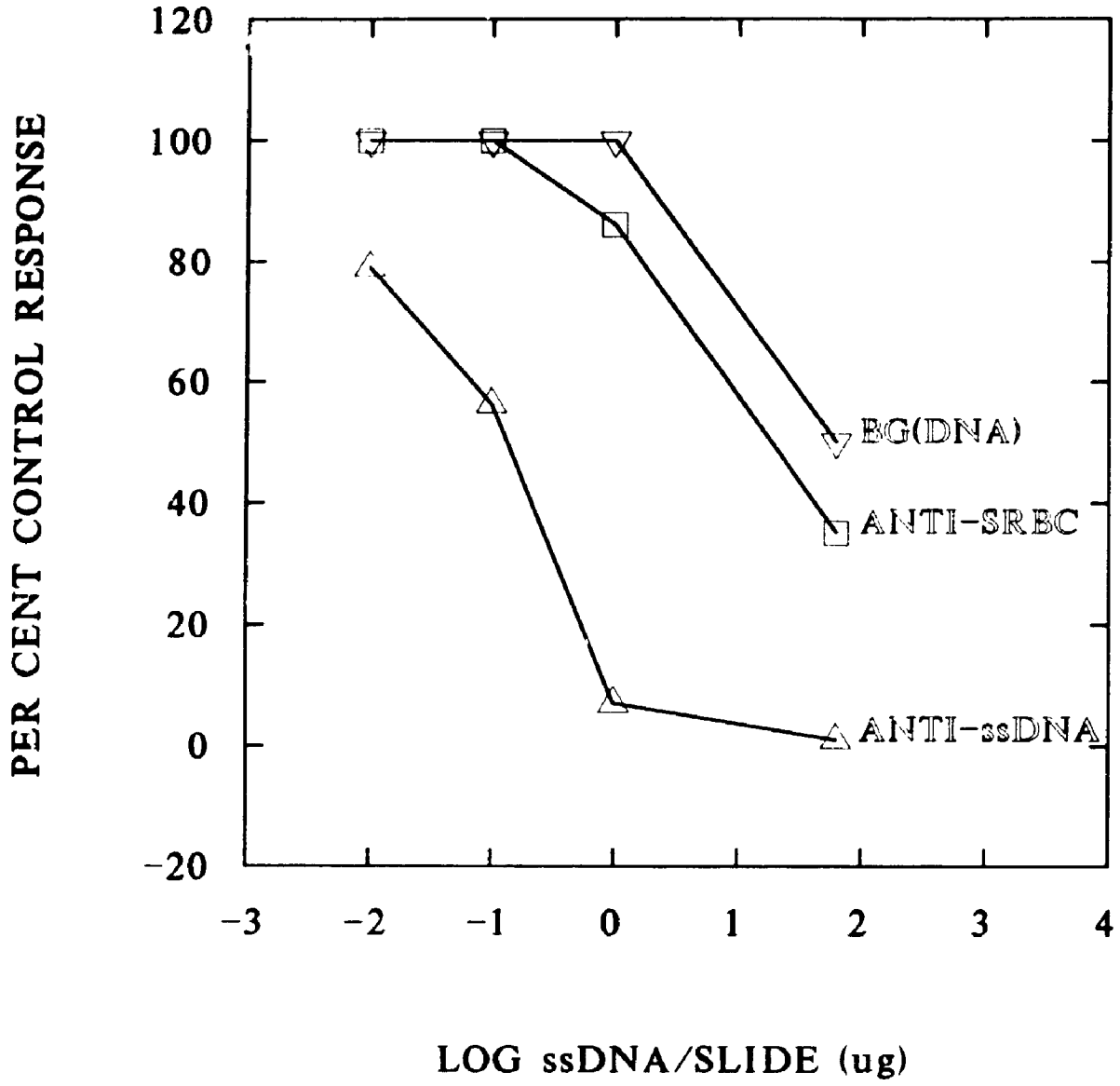

Figure 3.1. Antigenic specificity of the anti-ssDNA plaque assay: incorporation of ssDNA into agarose at time of assay inhibits the formation of anti-ssDNA-SRBC plaques as compared to anti-SRBC plaques from CBA spleen immunized *in vitro* with SRBC. Background anti-SRBC plaques were unaffected. Standard error for all points did not exceed 15%.

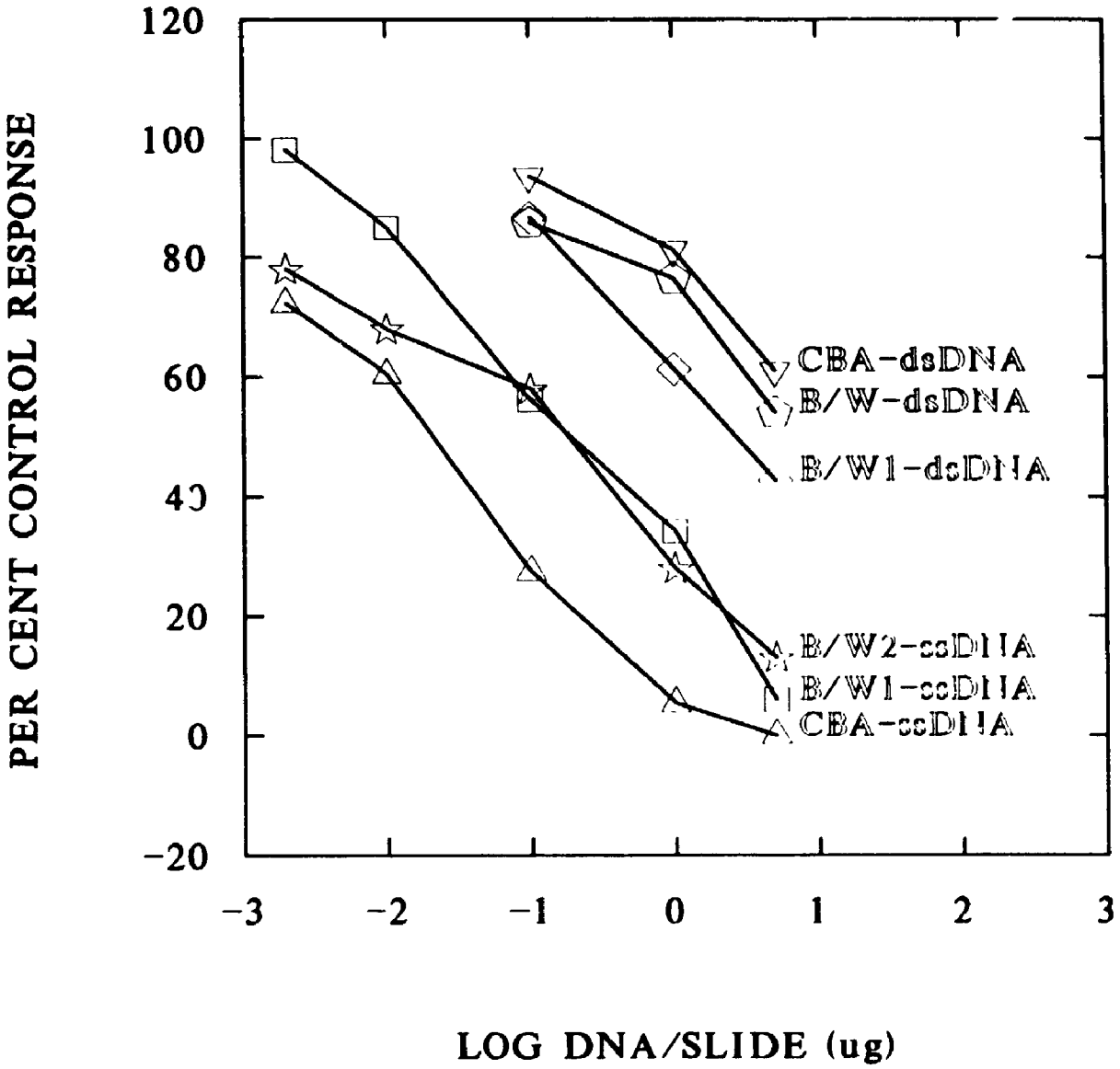

Figure 3.2. Fine specificity of the anti-ssDNA plaque assay: hibition of anti-ssDNA plaque formation by ssDNA incorporated into agarose at time of assay is more effective than dsDNA. Standard error for all points did not exceed 15%.

TABLE 3.3

Decreased Avidity of Spontaneous Autoantibody Forming Cells from an Autoimmune Strain of Mice - Preliminary Experiments[a]

Group[b]	PFC/10^6 ± SD (n)[c]	μg ssDNA for 50% inhib. ± SD (n)[d]
Athymic	667 ± 288 (8)	0.025 ± 0.016 (8)
CBA	629 ± 291 (9)	0.072 ± 0.047 (9)
Young B/W Pre-autoimmune	779 ± 383 (9)	0.510 ± 0.426 (9)
Old B/W Autoimmune	973 ± 709 (7)	0.913 ± 1.013 (7)

[a]Spleens from mice were cultured without adding DNA for 5 days and assayed for anti-single-stranded DNA antibody producing cells.
[b]Nudes, normal CBA, and NZB/NZW, all 6-8 weeks old, and 7-10 month old B/W mice were studied.
[c]Plaque forming cells/10^6 spleen cells recovered ± standard deviation (number in group).
[d]Amount of single-stranded soluble DNA required for 50% inhibition ± standard deviation (number). The avidity of B/W plaque forming cells was significantly lower than that for normal CBA and nude mice.

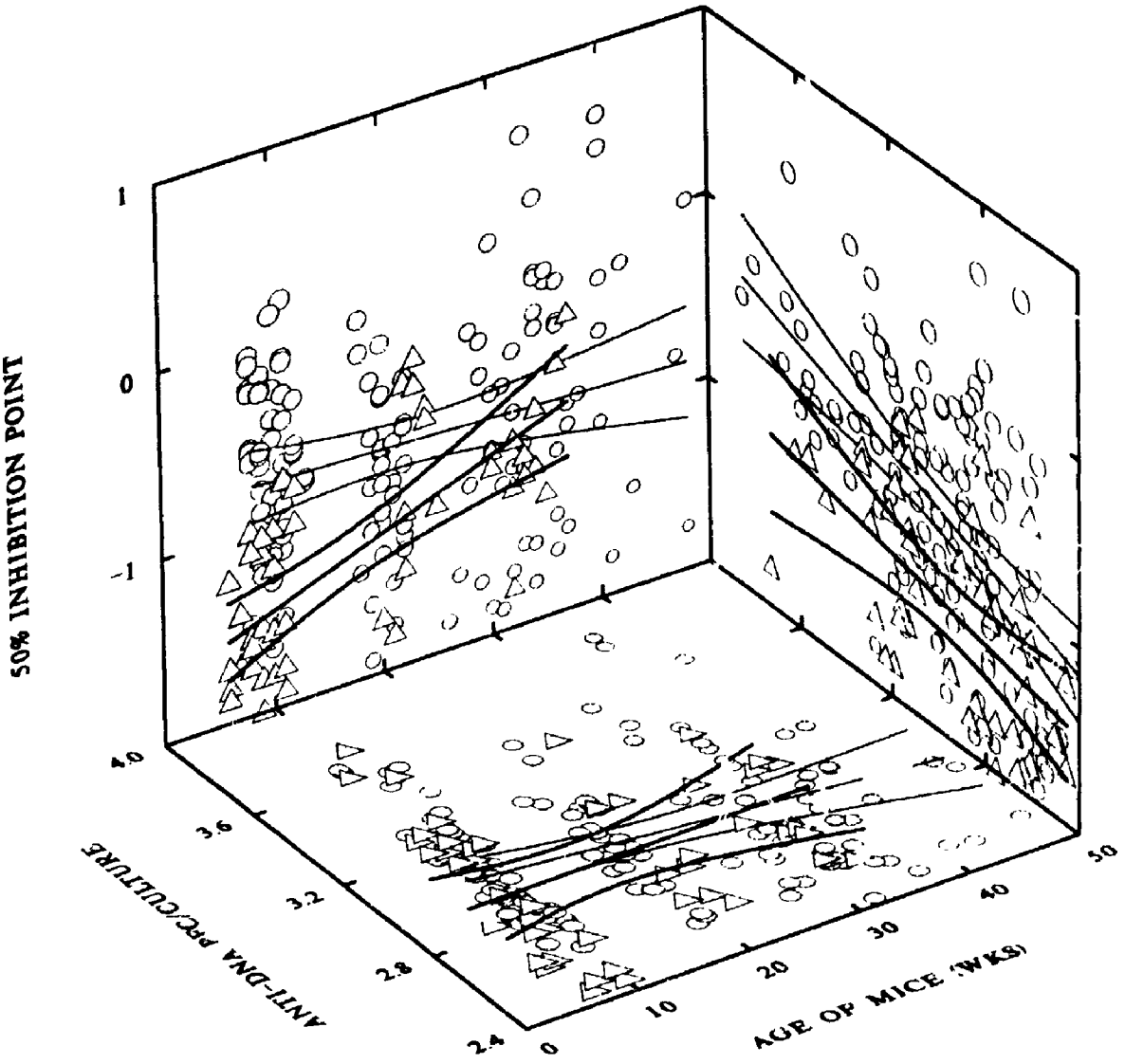

Figure 3.3. Three-way correlation between the quantity of free ssDNA required for 50% inhibition of plaques (Z-axis), the number of anti-ssDNA PFC per culture (Y-axis), and the age of the mice (X-axis). The means of non-autoimmune mice (triangles) are illustrated by thick straight lines bounded by bars of standard deviation. The means of mice of autoimmune background (circles) are illustrated by thin straight lines bounded by bars of standard deviation.

Furthermore, inefficient negative Fc signal transmission may be the prime deregulating element leading to conditions conducive for the emergence of clinical autoimmunity.

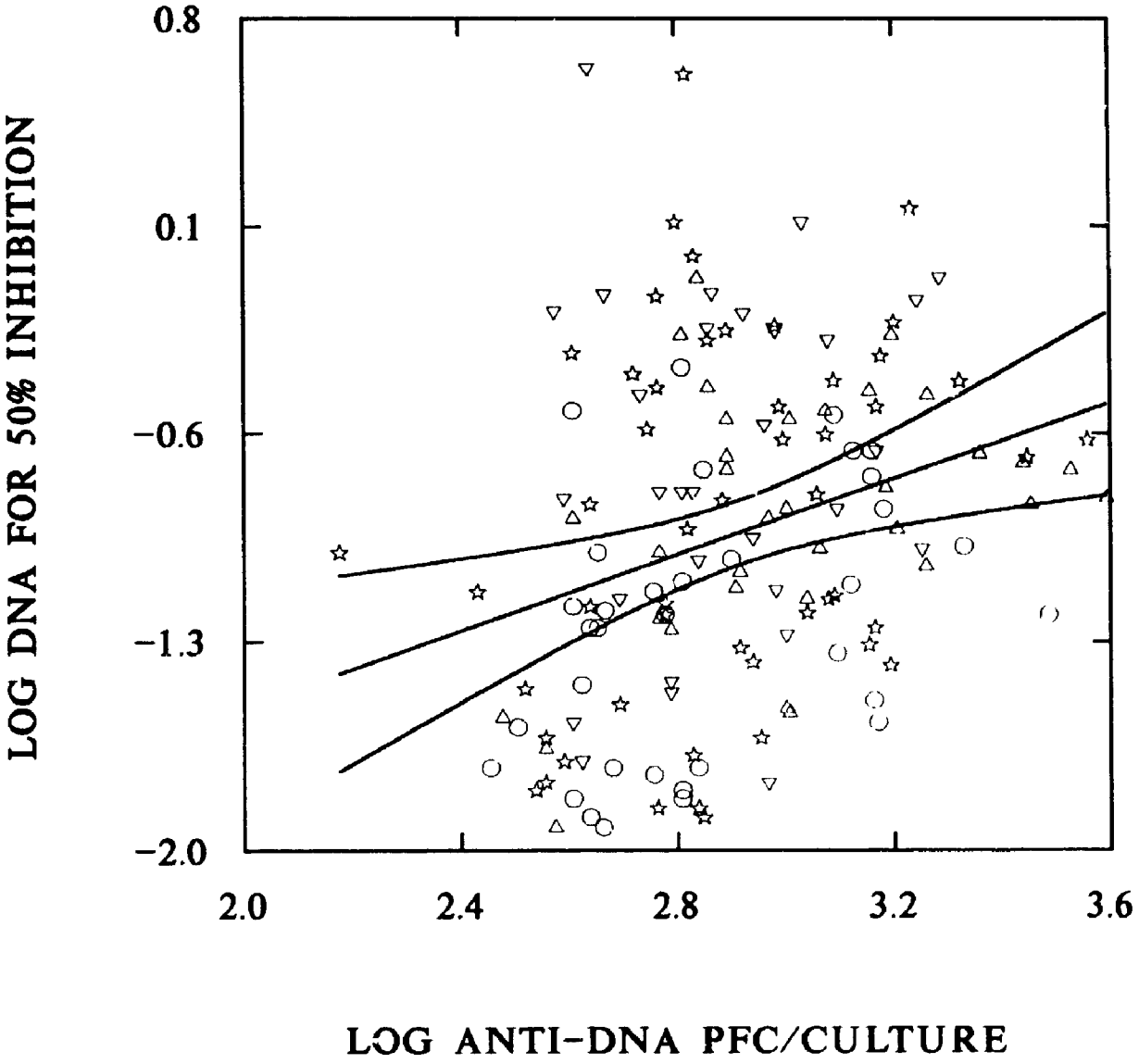

Figure 3.4. Correlation between amount of free ssDNA required for 50% inhibition of plaques (log, g/slide) and number of plaques per culture. Coefficient of correlation, r^2-value, is 0.09 ($p < 0.001$).

TABLE 3.4

Effect of Age and Mouse Strain on Anti-ssDNA Plaque Avidity

Strain	Age of Mouse (Weeks)		
	6 - 13	18 - 25	26 - 48
CBA	0.052 ± 0.009 (21)[a]	0.150 ± 0.043 (10)	0.101 ± 0.022 (12)
NZB/W	0.386 ± 0.089 (24)	0.190 ± 0.061 (12)	0.499 ± 0.159 (31)
NZB	0.308 ± 0.065 (13)	0.268 ± 0.118 (7)	0.722 ± 0.380 (11)
NZW	0.247 ± 0.068 (12)	0.149 ± 0.027 (13)	0.186 ± 0.058 (9)

[a] Plaque avidity is expressed as the concentration of DNA (μg/ml) required to cause 50% inhibition of the plaque count in the assay system. The numbers are given as the mean concentration of DNA ± standard error (number of mice/group).

TABLE 3.5

Effect of Age, Gender and Autoimmune Status on Anti-ssDNA Plaque Avidity

Status/Gender	Age of Mouse (Weeks)		
	6 - 13	18 - 25	26 - 48
Non-autoimmune			
Male	0.059 ± 0.014 (11)[a]	0.085 ± 0.030 (7)	0.075 ± 0.014 (6)
Female	0.045 ± 0.014 (10)	0.303 ± 0.066 (3)	0.127 ± 0.042 (6)
Autoimmune			
Male	0.354 ± 0.066 (31)	0.153 ± 0.039 (18)	0.442 ± 0.109 (31)
Female	0.293 ± 0.075 (18)	0.238 ± 0.064 (14)	0.569 ± 0.281 (20)

[a] Plaque avidity is expressed as the concentration of DNA (μg/ml) required to cause 50% inhibition of the plaque count in the assay system. The numbers are given as the mean concentration of DNA ± standard error (number of mice/group).

TABLE 3.6

Effect of Age and Mouse Strain on Anti-ssDNA Plaque Production

Strain	Age of Mouse (Weeks)		
	6 - 13	18 - 25	26 - 48
CBA	1493 ± 303 (21)[a]	1074 ± 259 (10)	937 ± 150 (12)
NZB/W	1663 ± 273 (24)	1094 ± 115 (12)	1423 ± 296 (31)
NZB	909 ± 74 (13)	1029 ± 228 (7)	649 ± 127 (11)
NZW	1525 ± 303 (12)	1308 ± 243 (13)	812 ± 129 (9)

[a] Anti-DNA antibody forming cells are expressed as plaques/culture. The numbers are given as the mean plaque count ± standard error (number of mice/group).

TABLE 3.7

Effect of Age and Mouse Strain on Viable Cells Recovered from Cultures

Strain	Age of Mouse (Weeks)		
	6 - 13	18 - 25	26 - 48
CBA	3.39 ± 0.22 (21)[a]	3.01 ± 0.18 (10)	2.71 ± 0.18 (12)
NZB/W	3.04 ± 0.19 (24)	2.62 ± 0.21 (12)	2.74 ± 0.18 (31)
NZB	2.88 ± 0.13 (13)	2.72 ± 0.24 (7)	2.73 ± 0.17 (11)
NZW	2.87 ± 0.21 (12)	2.84 ± 0.17 (13)	3.05 ± 0.27 (9)

[a] Viable cell recoveries are expressed as cells$\times 10^6$/culture.
The numbers are given as the mean cell count ± standard error (number of mice/group).

TABLE 3.8

Effect of Age and Mouse Strain on Spontaneous Anti-SRBC Plaque Production

Strain	Age of Mouse (Weeks)		
	6 - 13	18 - 25	26 - 48
CBA	3.10 ± 1.19 (21)[a]	2.05 ± 0.65 (10)	2.67 ± 0.58 (12)
NZB/W	5.75 ± 1.13 (24)	10.88 ± 1.83 (12)	13.48 ± 1.66 (31)
NZB	13.73 ± 2.23 (13)	15.07 ± 2.80 (7)	10.55 ± 2.28 (11)
NZW	1.54 ± 0.32 (12)	2.92 ± 0.62 (13)	5.28 ± 1.42 (9)

[a] Spontaneous anti-sheep erythrocyte antibody forming cells are expressed as plaques/slide. The numbers are given as the mean plaque count ± standard error (number of mice/group).

TABLE 3.9

Comparison of RF Concentrations as Determined by Our ELISA Versus that of Contributing Laboratory

Group	Our value[a]	Coulie's value[b]
RF1	7.02	9.0
RF2	5.60	6.5
RF3	3.52	3.5
RF4	3.20	3.5
RF5	2.80	4.0

[a]In μg/ml as measured on goat-anti-mouse F(ab')$_2$-coated plate, using goat-anti-mouse μ as a developing reagent. Only background readings were obtained with goat-anti-mouse IgG.
[b]Values in μg/ml given by Dr. P. Coulie.

TABLE 3.10

Immunoglobulin and Anti-DNA Levels in CBA and NZ Mice

STRAIN	TOTAL IgM	TOTAL IgG	ANTI ssDNA IgM	ANTI ssDNA IgG
Culture Supernatants (μg/ml)				
CBA	11.5(22)	0.062(17)	3.776(28)	0.008(17)
NZB/W	7.5(42)	0.474(29)	2.461(32)	0.141(25)
NZB	17.2(25)	0.256(17)	2.396(23)	0.023(14)
NZW	10.7(28)	0.612(20)	3.659(27)	0.014(18)
Serum (μg/ml)				
CBA	546.1(17)	2659.5(5)	260.8(15)	6.3(6)
NZB/W	380.3(18)	1722.5(7)	466.2(21)	1257.4(7)
NZB	1077.7(14)	5013.5(3)	1000.8(14)	177.1(7)
NZW	184.1(16)	1616.0(6)	172.5(21)	10.5(8)

[a] Concentration of total IgM or IgG, or anti-ssDNA (IgM or IgG), in μg/ml. Numbers in brackets indicate number of mice tested. Sham supernatants (i.e. culture medium alone) gave background readings.

TABLE 3.11

Rheumatoid Factor Levels in CBA and New Zealand Mice

STRAIN	Fc	IgG	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3
Culture Supernatants (μg/ml)						
CBA	.098* (26)	.084 (26)	.093 (24)	.214 (26)	.080 (18)	.116 (26)
NZB/W	.103 (38)	.178 (38)	.124 (36)	.122 (36)	.350 (31)	.108 (38)
NZB	.719 (25)	.413 (25)	.159 (25)	.171 (25)	1.099 (22)	.138 (25)
NZW	.172 (27)	.076 (28)	.087 (27)	.113 (28)	.098 (22)	.088 (27)
Serum (μg/ml)						
CBA	52.3 (20)	146.1 (18)	45.8 (18)	47.6 (17)	61.2 (15)	67.1 (18)
NZB/W	104.4 (22)	93.3 (22)	109.7 (23)	93.4 (23)	47.6 (22)	84.4 (22)
NZB	955.1 (21)	2450.7 (18)	621.0 (18)	686.9 (18)	531.4 (16)	2031.4 (18)
NZW	12.2 (26)	17.6 (19)	16.3 (18)	12.0 (18)	15.0 (17)	18.6 (19)

* Concentration of indicated specificities of rheumatoid factor in μg/ml. Numbers in brackets indicate number of mice tested. Sham supernatants (i.e. culture medium alone) gave background readings.

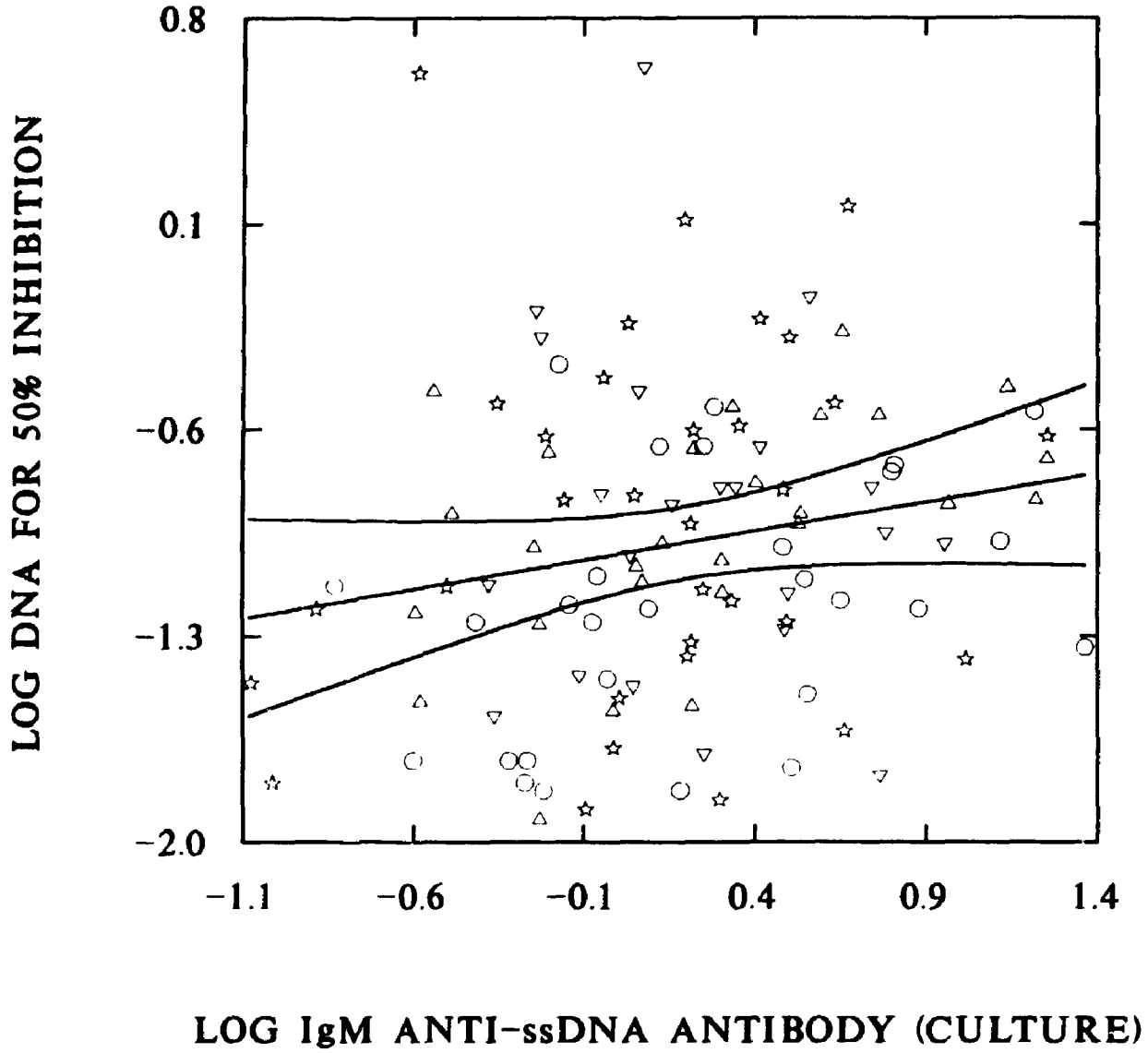

Figure 3.5. Correlation (r^2 = 0.04, p = 0.05) between quantity of free ssDNA (log μg/slide) required for 50% inhibition of plaques and the concentration of IgM-anti-ssDNA Ab (log μg/ml) in culture supernatant. Each point represents one mouse and each strain is represented by a different symbol: CBA (circles); B/W (stars); NZB (upright triangles); NZW (upside-down triangles).

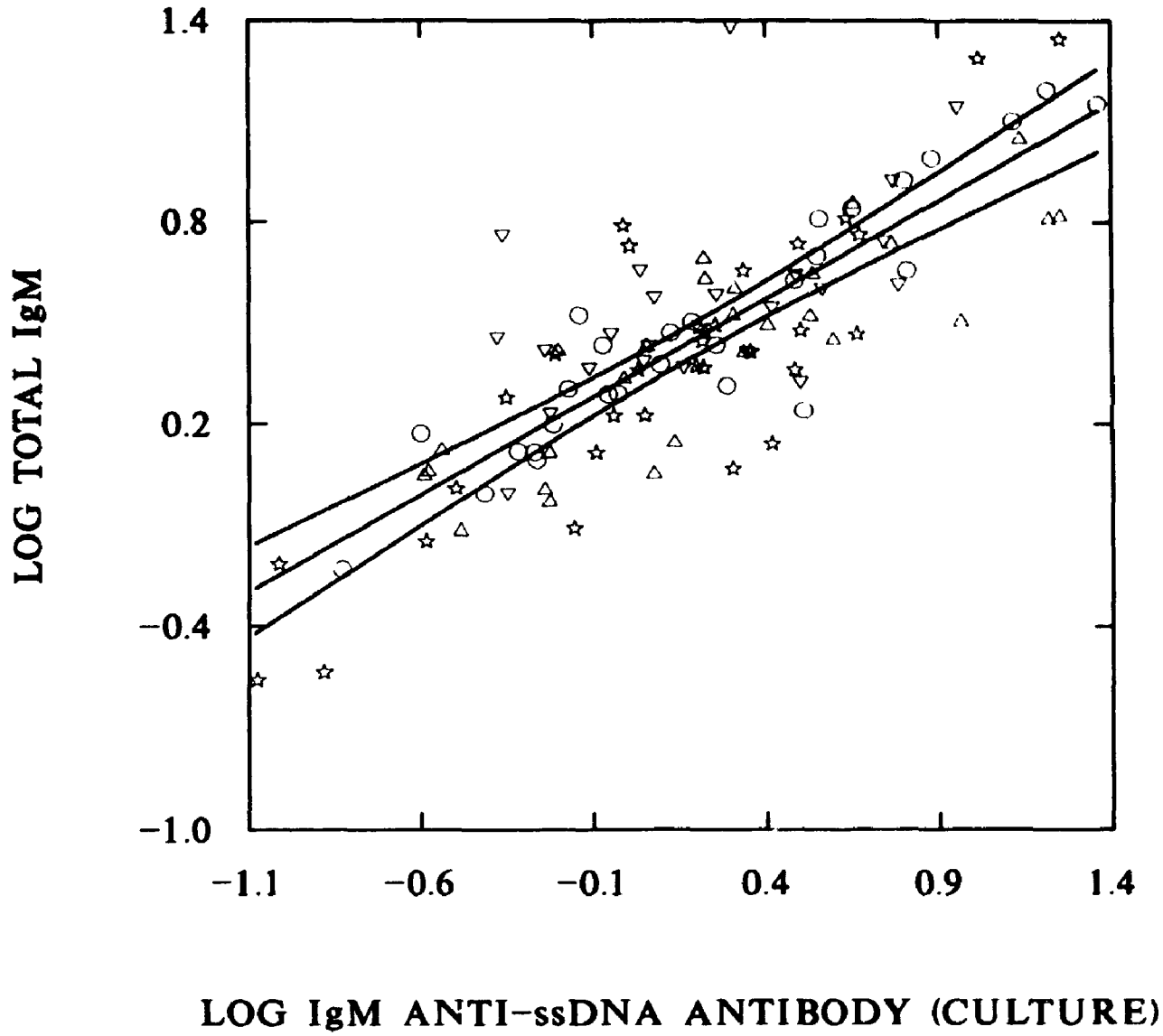

Figure 3.6. Positive correlation ($r^2 = 0.67$, $p < 0.001$) between level of total IgM and IgM-anti-ssDNA Ab (both as log μg/ml) in culture supernatants. Symbols as per Figure 3.5.

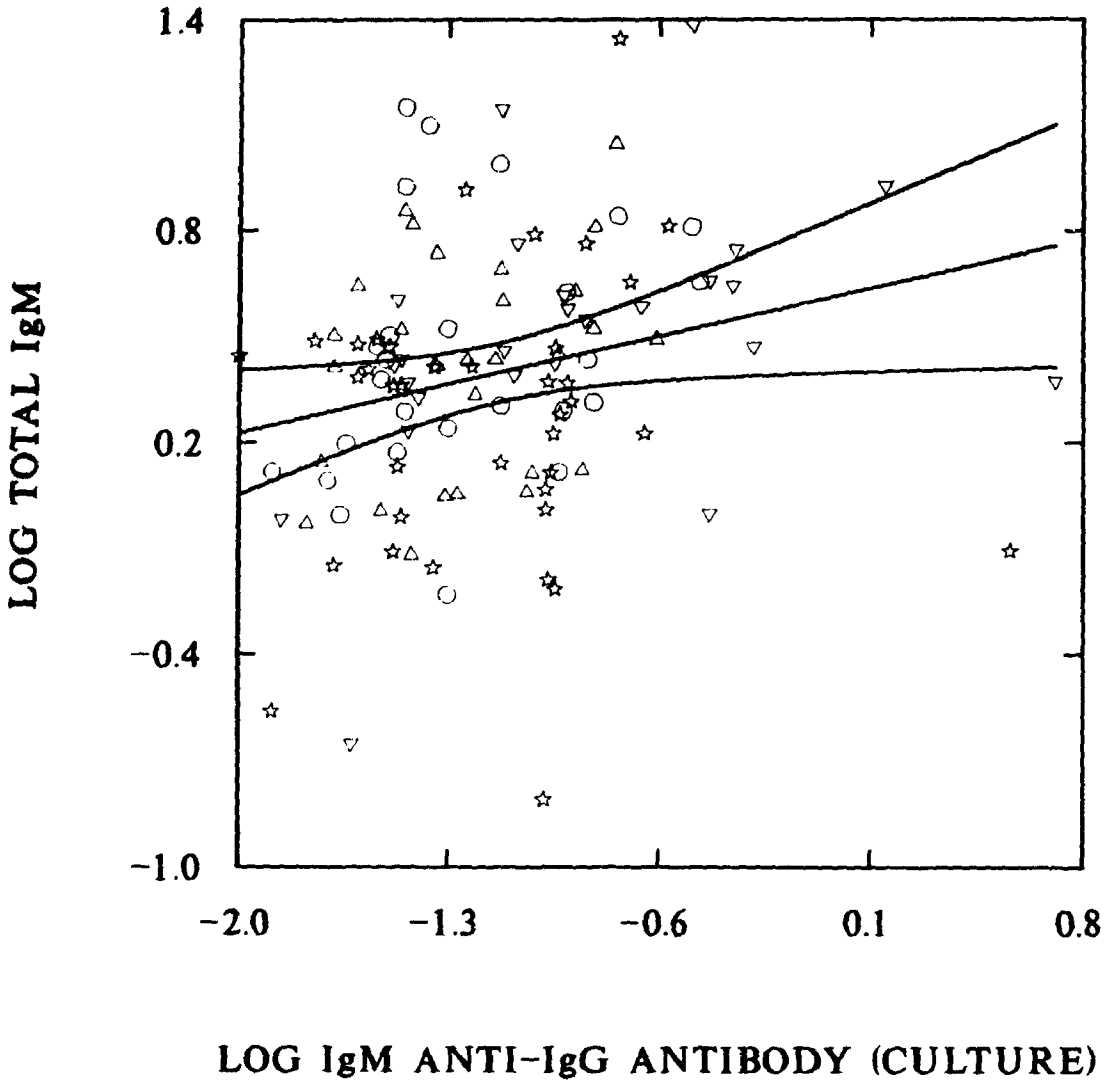

Figure 3.7. Lack of correlation (r^2 = 0.003, p = 0.7) between total IgM and RF levels (both as log μg/ml) in culture supernatants. Symbols as per Figure 3.5.

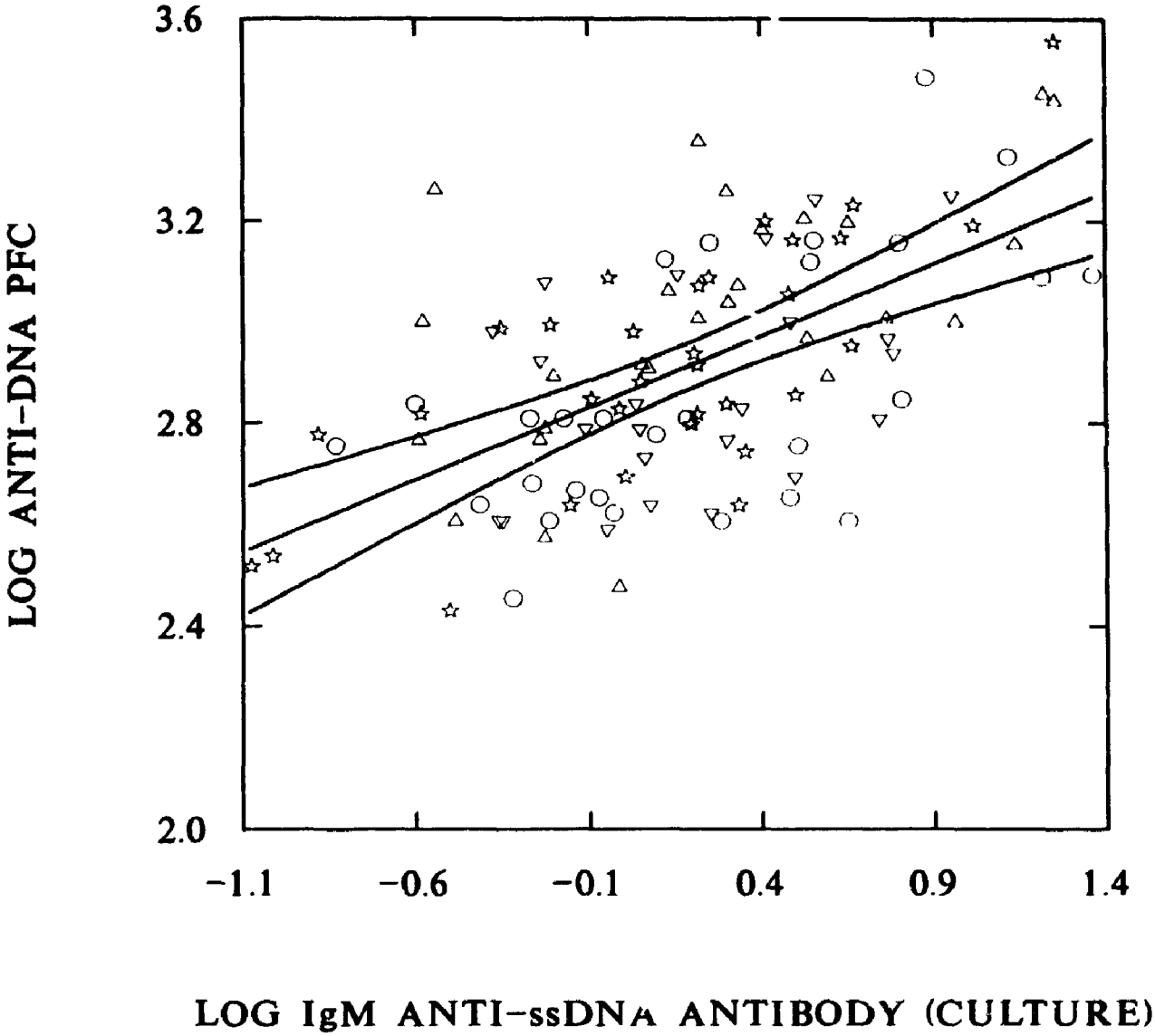

Figure 3.8. Positive correlation ($r^2 = 0.36$, $p < 0.001$) between number of anti-DNA PFC/culture and level of IgM-anti-ssDNA Ab (log μg/ml) in culture. Symbols as per Figure 3.5.

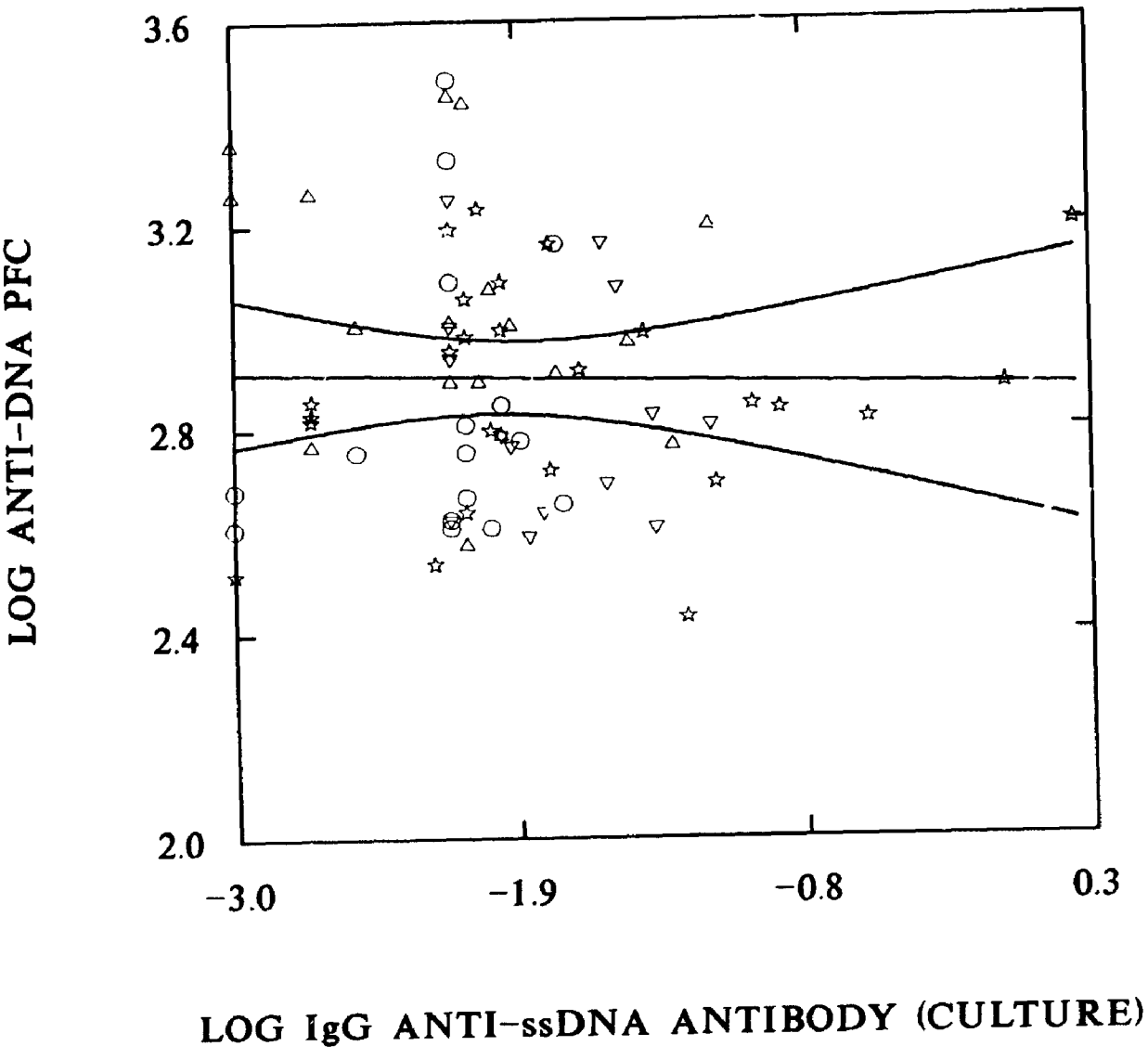

Figure 3.9. Lack of correlation (r^2 = 0.001, p = 0.8) between number of anti-DNA PFC/culture and level of IgG-anti-ssDNA Ab (log μg/ml) in culture. Symbols as per Figure 3.5.

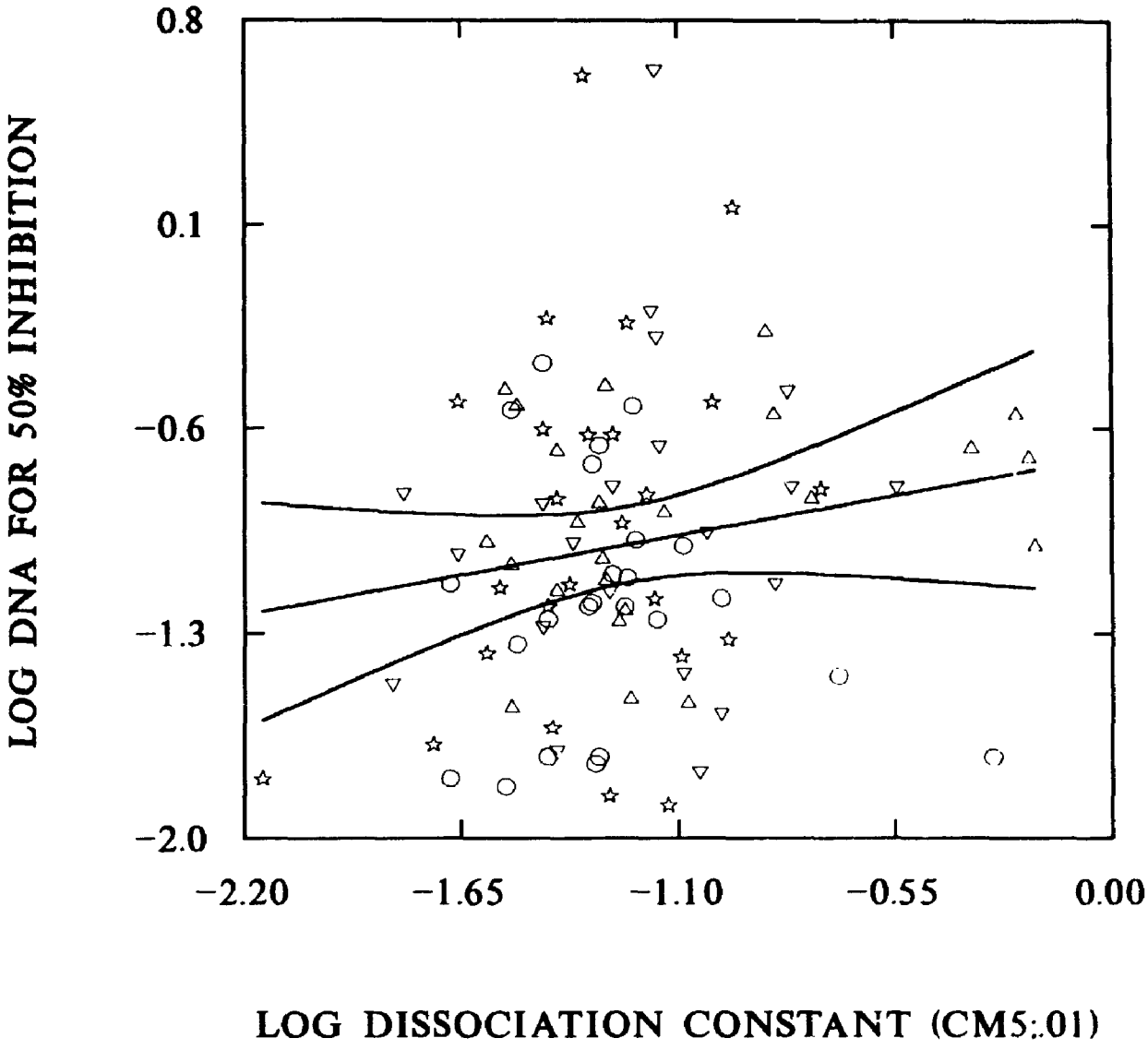

Figure 3.10. Correlation (r^2 = 0.03, p = 0.1) between quantity of free ssDNA (log μg/slide) required for 50% inhibition of plaques and log of dissociation constant (x 10^{-10}/mole) of IgM-anti-ssDNA Ab in culture as determined by ELISA. Increase in amount of free ssDNA and K_D both indicate lower antibody avidity. Symbols as per Figure 3.5.

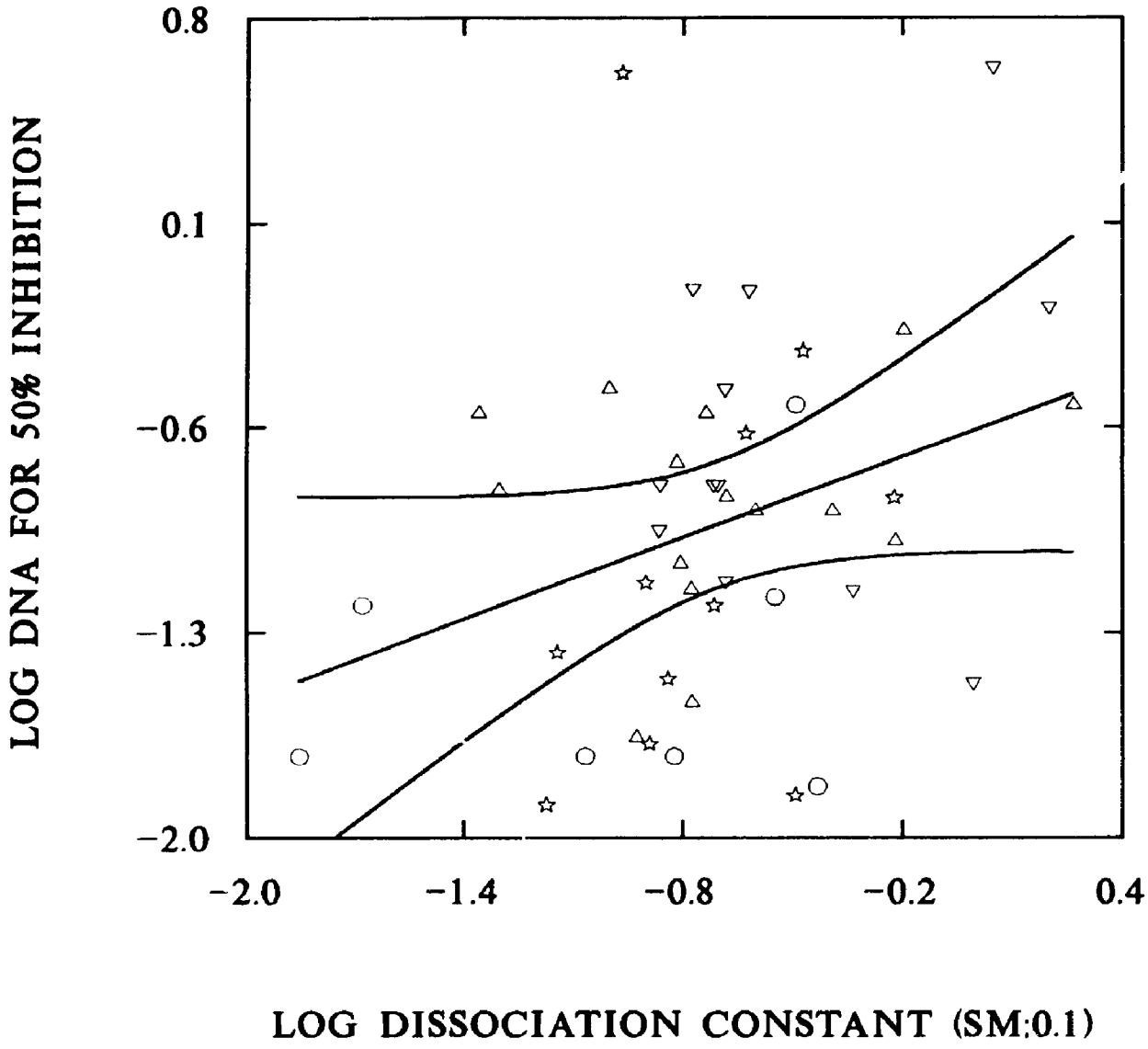

Figure 3.11. Correlation (r^2 = 0.07, p = 0.08) between quantity of free ssDNA (log μg/slide) required for 50% inhibition of plaques and log of dissociation constant (x 10^{-10}/mole) of IgM-anti-ssDNA Ab in serum as determined by ELISA. Symbols as per Figure 3.5.

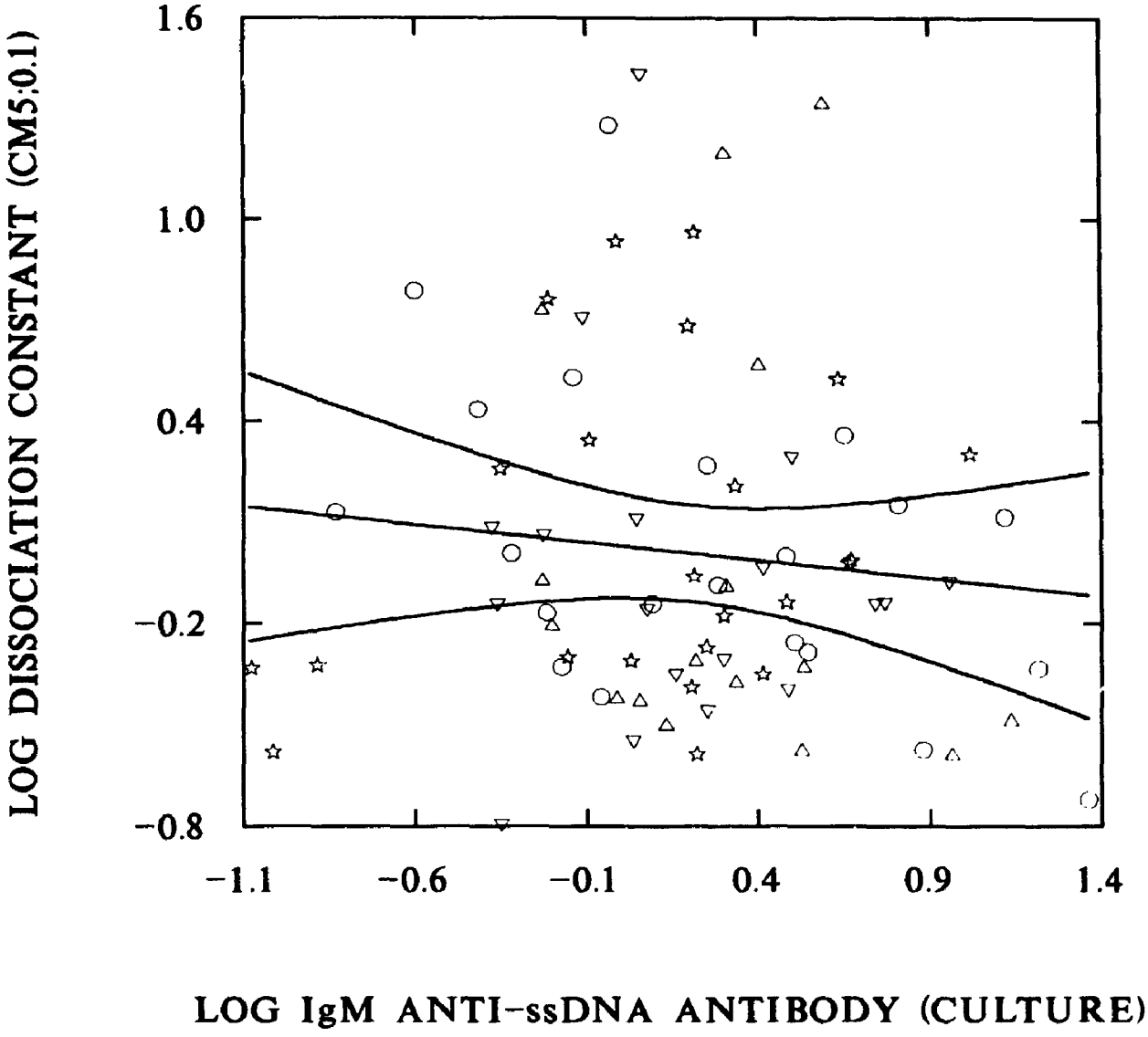

Figure 3.12. Lack of correlation (r^2 = 0.01, p = 0.4) between log of K_D (x 10^{-10}/mole) and level of IgM-anti-ssDNA Ab (μg/ml) in culture. Symbols as per Figure 3.5.

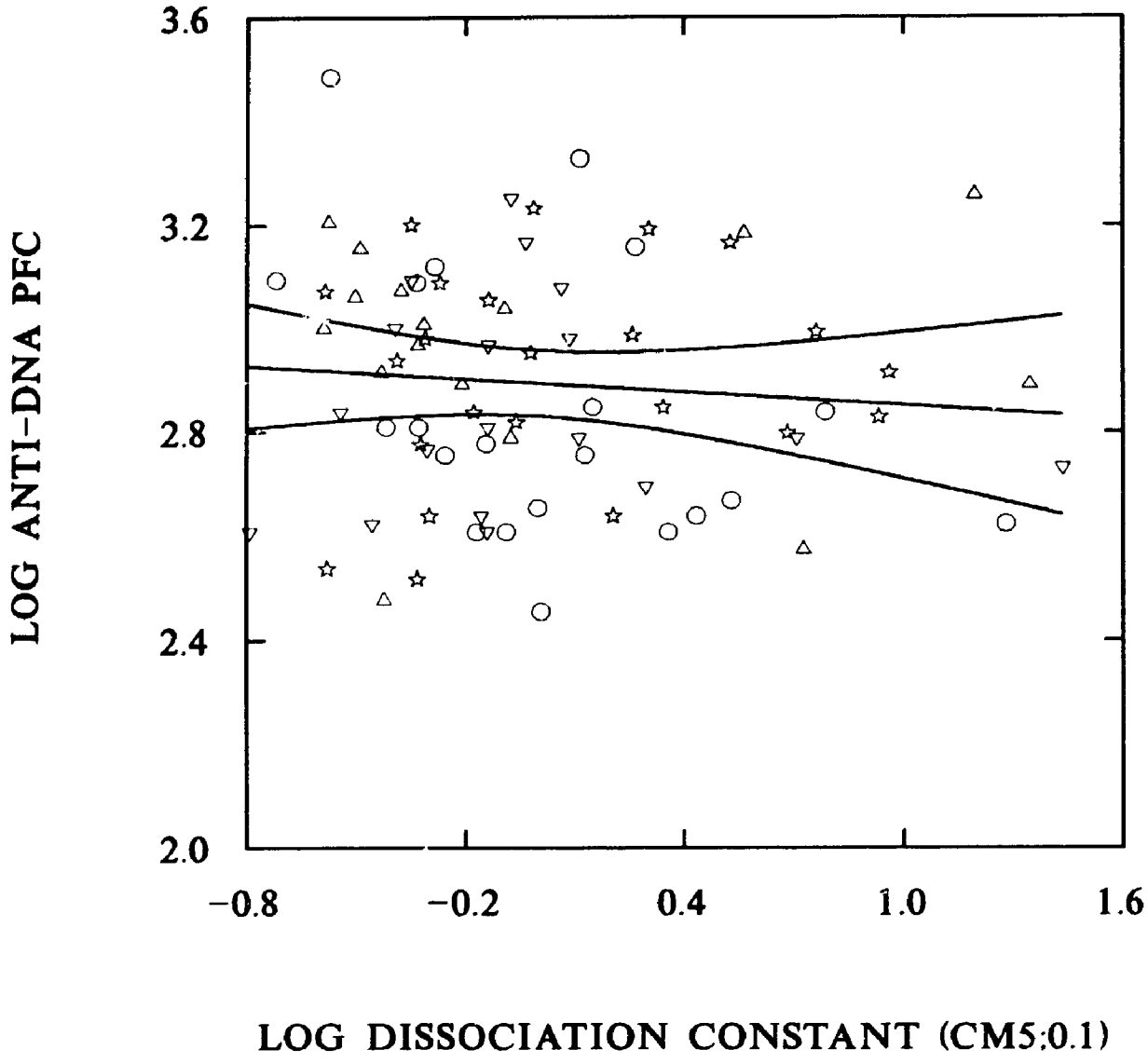

Figure 3.13. Lack of correlation (r^2 = 0.01, p = 0.4) between number of anti-ssDNA PFC/culture and log of K_D (x 10^{-10}/mole) of IgM-anti-ssDNA Ab in culture supernatant. Symbols as per Figure 3.5.

3.4 DISCUSSION

The New Zealand mouse is used as an animal model for the study of autoimmune disorders and it closely parallels human systemic lupus erythematosus. One of the major stigmata of lupus, as in many other systemic autoimmune syndromes, is the production of anti-DNA antibodies. The final surge of the autoimmune process entails a switch from IgM to predominant production of cationic, complement-fixing IgG-anti-DNA antibodies which are quick to localize on the anionic glomerular basement membrane and effect the tissue damage and ensuing fatal glomerulonephropathy. Attempts to isolate the "aberrant" stimulating autoantigen have proved frustrating since cells with specificity for all autoantigens associated with autoimmune disease exist in the healthy immunocompetent host (Klinman *et al.*, 1988a; 1988b). Although it is known that IgM-anti-ssDNA antibodies are produced in the absence of lupus (Sawada *et al.*, 1977), a comparison between normal and autoimmune-prone animals in both the capacity for autoantibody production and the avidity for autoantigen prior to binding and sequestration has not been done until now.

The major finding of this study is that an end-product IgM antibody of anti-ssDNA PFC, generated *in vitro*, shows a higher avidity towards ssDNA in normal mice than does a similar antibody formed by cells from autoimmune-prone mice. This higher avidity is seen predominantly in cells from *young, non-autoimmune-prone* mice; the avidity tends to decrease with age. Therefore, young non-autoimmune-prone mice have, within their repertoire of responding B cells, what they are not supposed to have, according to the clonal deletion/abortion/anergy theory of non-reactivity to self (Burnet, 1957; Nossal and Pike, 1975; 1980; Nossal, 1986;): high avidity anti-self recognition elements which are deregulated *in vitro*. In fact, the level of anti-self recognition elements at the IgM stage of B cell activation is higher in avidity of PFC and equal to, or higher, in amount of *in vitro* secreted

anti-ssDNA antibody than that seen in autoimmune mice. Since this stage of B cell activation must be considered well beyond an early stage in B cell ontogeny, both in terms of a differentiation pathway and in terms of the life span of the host, these results contradict an obligatory component of the clonal deletion/abortion theory (Burnet, 1957; Nossal, 1986; 1988).

An argument raised against the use of ssDNA as a representative self-antigen is that it is a degraded form of a self component which, moreover, is an internal self constituent of cells and, hence, has been referred to as endogenously generated non-self (Cohn, 1989). However, this definition can be applied to all autoantigens since they are all ultimately derived from inside the cells which produced them. Furthermore, ssDNA, along with other "culprit" self-antigens, is produced continuously from early fetal life as a result of the specific cellular differentiation of erythrocytes and as a general process of degradation of effete cells. The spontaneous generation of anti-ssDNA antibodies *in vitro* must signify that ssDNA is perceived *in vivo* but that responses to it are normally controlled.

The anti-ssDNA antibody which is most associated with SLE is of the IgG isotype (Steward and Hay, 1976; Papoian *et al.*, 1977). Since the culture system employed supported a predominantly IgM response, it is mainly an *in vitro* IgM PFC response to ssDNA which has been studied. A study of IgM anti-ssDNA is relevant to the question of what type of aberration in self-nonself discrimination is conducive for the development of clinical autoimmunity. Studying B cell autoreactivity, in the stage prior to production of pathogenic IgG antibody, will help us to understand the nature of the step which malfunctions.

Since normal animals can produce IgM autoantibody and the pathogenic IgG autoantibodies occur in autoimmunity, it is conceivable that self-nonself discrimination occurs, in normal animals, during the switch from IgM to IgG, and defective control of this switch occurs in autoimmune-prone animals. This must be

the meaning of the greater capacity to form high avidity IgM anti-ssDNA PFC and low concentrations of IgG antibodies in normals, and the reverse in autoimmune-prone animals. High avidity IgG autoantibody in low concentrations normally serves as a negative feedback regulator (Sinclair and Panoskaltsis, 1987b; Dziarski, 1987), thus stopping the continued differentiation of autoreactive B cells towards massive, and damaging, IgG autoantibody production. Hence, a B cell with reactivity to self is normally stopped by this *autologously* produced IgG autoantibody.

In non-autoimmune animals with little autoantibody, the majority of autoantibody is of the IgG class (Avrameas *et al.*, 1981; 1983; Guilbert *et al.*, 1982; Dighiero *et al.*, 1985a; 1985b; Ericsson *et al.*, 1985; Holmberg and Coutinho, 1985; Ruf *et al.*, 1985; Avrameas, 1986; Cohen and Cooke, 1986; Mahana *et al.*, 1988; Matsiota *et al.*, 1988; Nakamura *et al.*, 1988; Piechaczyk *et al.*, 1985; 1987; 1989; Bouanani *et al.*, 1989). This is contrary to what has been claimed (Nossal, 1989; McHeyzer-Williams and Nossal, 1989). When non-autoimmune animals produce autoantibody in larger amounts which is detected by insensitive assays, it is often of the IgM isotype. This is similar to the event, described here, which takes place *in vitro*. In autoimmune animals, IgG autoantibody is overproduced (Cohen and Cooke, 1986; Steward and Hay, 1976; Papoian *et al.*, 1977). Hence, there are three conditions with respect to autoantibody production:- 1) a minimal IgG response which controls autoantibody production of all isotypes, 2) an increased IgM autoantibody response indicative of reduced regulation by constitutive IgG autoantibody which occurs normally and is of limited pathological significance, and 3) an increased production of IgG autoantibody due to a pathological loss of the IgM-IgG switch control.

High affinity antibodies are more effective suppressors of the humoral response (Walker and Siskind, 1968; Heyman and Pilstrom, 1988). Marked IgG Fc-

mediated immunosuppression is correlated with high avidity IgG antibodies and not with the IgG subclass (Bruggemann and Rajewsky, 1982; Heyman and Wigzell, 1984). This correlates with the low specificity of Fc-receptors on B cells for subclasses of IgG (Heyman, 1989). That high avidity antibody-producing cells are more easily rendered tolerant than low avidity cells (Theis and Siskind, 1968) suggests that these cells are more likely to form inactivating crosslinks between antigen-receptors and Fc-receptors.

Fc-mediated end product feedback by antibody to antigen is the rate-limiting, early expressed mechanism for controlling primary T cell dependent antibody responses (Sinclair and Panoskaltsis, 1987a; and results of this thesis). This immunoregulatory mechanism, which is modified by T cell activity, shows many forms of derangement in autoimmunity which include abnormal glycosylation of the Fc portion of IgG in rheumatoid arthritis (Parekh *et al.*, 1985; Rook, 1988) which disturbs the ability of the Fc portion to immunosuppress (Heyman *et al.*, 1985), various defects in B cell Fc-receptors in NZB mice (Uher and Dickler, 1986b), and the production of various agents which block Fc signals, such as rheumatoid factor (RF) (Vaughan, 1979), microbial products (Mouritsen, 1986) and T cell factors (Phillips *et al.*, 1988, O'Garra *et al.*, 1987; Laszlo and Dickler, 1988). Therefore, in autoimmunity, defects in the Fc portion of antibody, in Fc receptors, and in the regulation of Fc signal blockers (such as RF and other endogenous and exogenous Fc blocking agents) have been demonstrated. These studies leave little doubt that a major defect in the pathogenesis of autoimmunity is in the Fc signalling mechanism and the lack of control at the IgM-IgG switch point.

In non-autoimmune-prone animals, autoreactive B cells are downregulated by low levels of autologous IgG and prevented from progressing through the IgM-IgG switch. Therefore, there would be an accumulation of cells capable of IgM synthesis. Since the B cells with high avidity antigen-receptors most easily succeed

in switching, when they are not inhibited by Fc signals, cells capable of producing high avidity IgM antibodies would be expected to accumulate in the presence of Fc-dependent IgG antibody end product feedback. The studies in this chapter have addressed the accumulation of cells with this capacity in normal mice, a process which is missing in autoimmune-prone mice and, hence, the avidity of IgM antibody in the latter mice is lower.

Many investigators have studied the relationship between anti-DNA antibody avidity (or affinity) and severity of disease both in humans and in murine models of SLE, but with contradictory results (reviewed in the introduction). These studies involved the examination of serum and/or glomerular eluate antibodies. A general problem in these studies is that what is measured may not reflect what is produced (Cronin *et al.*, 1986), due to complexing of antibody with antigen and possible subsequent complement fixation and immune complex deposition or clearance. Another consideration, this time relating to the interpretation of the results given here, is whether the antibody measured is produced by cells which are situated before or after the IgM-IgG switch which is abnormally active in autoimmunity. In reviewing the literature, it was not possible to compare the normal (or mildly affected) with diseased animals/individuals for the amounts and avidities of *both* IgM and IgG autoantibodies produced; this point requires further study, this one being an initial attempt at this approach.

The results complement other observations on the genesis of autoimmunity including the polyclonal nature of B cell responses in autoimmune prone animals (Klinman and Steinberg, 1987b; Dar *et al.*, 1988), the presence of common and/or unique idiotypes in both normal and autoimmune animals (Solomom *et al.*, 1983; Cairns, E. *et al.*, 1984; Rauch *et al.*, 1985; Hahn and Ebling, 1987; Ebling *et al.*, 1988; Madaio *et al.*, 1987; Matsiota *et al.*, 1987; Kaushik *et al.*, 1988; Klinman *et al.*, 1988a; Datta *et al.*, 1987; Yoshida *et al.*, 1987), and, since Fc-signalling is influenced by

regulatory T cells and possibly by the mode of antigen presentation (Sinclair and Panoskaltsis, 1988), the presence of defects both in the B cell lineage (Yoshida *et al.*, 1987) and external to it (Hirano *et al.*, 1987; Ishigatsubo *et al.*, 1987; Klinman and Steinberg, 1987a; Klinman *et al.*, 1988b) including defects in suppressor T cells and T cell tolerance (Yunis, 1967; Wick *et al.*, 1970; Harris *et al.*, 1982; Cairns, L. *et al.*, 1986; Taguchi and Nishizuka, 1987; Boguniewicz *et al.*, 1989). It is possible that the central process which becomes deranged in autoimmunity, thus giving rise to the observed defects in autoimmune disease, is Fc signalling. If a major defect in autoimmunity is an inadequate control in IgM to IgG switching, this abnormality is more in keeping with deficiencies in Fc signalling than to considerations relating to incorporation of lymphocytes into an idiotypic network (Stewart *et al.*, 1989), whatever this concept may mean. Lastly, the recent reports (Mahana *et al.*, 1989a; 1989b), demonstrating that newborns are able to generate immune responses to self antigens, can be interpreted as an immaturity in Fc signalling mechanisms, which, once matured, utilize early anti-self IgG responses to attain quiescence to self.

CHAPTER 4 EFFECT OF RF ON AUTOANTIBODY AVIDITY -- INTERFERENCE WITH, OR LACK OF, EFFECTIVE NEGATIVE Fc SIGNALLING AFFECTS AFFINITY MATURATION OF RESPONSE

4.1 INTRODUCTION

It has been demonstrated that the *in vitro* spontaneous production of anti-ssDNA antibodies by murine spleen cells can be suppressed up to 100% in a dose-dependent fashion by the addition of high molecular weight ssDNA into culture (Roder *et al.*, 1978b). The previous authors proposed "receptor blockade" (Schrader and Nossal, 1974; Klaus, 1976) as the mechanism of the "regulation" but since there was evidence to show that the response against a foreign antigen was regulated at the onset by negative Fc-signalling (Sinclair and Panoskaltsis, 1986a; 1986b; 1987a; 1987c; and the results of this thesis), it would be difficult not to attempt to determine if autoimmune responses could also be regulated by negative Fc signals. One could demonstrate Fc-mediated regulation of the spontaneous *in vitro* anti-ssDNA response by "suppressing" this autoimmune PFC response by the addition of high MW ssDNA to murine spleen cell cultures and preventing suppression by blocking endogenous negative Fc signals with the simultaneous (concomitant) addition of rheumatoid factor (RF). Not only would it be shown that an autoimmune response could be suppressed by autoantigen/secreted-antibody complexes via an endogenous Fc-dependent mechanism, but also that RF could affect this mechanism and, hence, link ineffective negative Fc signalling to the dysregulation of an autoimmune response.

4.2 METHODS

T cell depletions were performed as described in section 2.2.5. Cell proliferation in response to mitogens was monitored as per section 2.2.6. All other materials and methods used are as described in Chapter 3.2 with the addition of the following modification for the inhibition of IgM plaques by Con A (Nordin *et al.*, 1969): the slides were incubated with Con A at a concentration of 0.4mg/ml BSS for one hour at 37^{o}C and then the slide trays were washed or the slides were put onto fresh trays. This Con A step preceded the incubation with facilitating serum.

4.3 RESULTS

4.3.1 Inability to Suppress Anti-ssDNA Response with High Molecular Weight DNA

Many attempts were made to suppress the *in vitro* anti-ssDNA PFC response as described by Roder *et al.*, 1978b. It can be seen in Table 4.1 that addition of increasing doses of ssDNA to cultures of spleen cells from either CBA/J mice or B/W mice could not suppress the anti-ssDNA response. The dose-range of ssDNA used (1 μg to 100 μg/culture) should have resulted in at least some suppression, if indeed the "receptor-blockade" mechanism were operating, since 100% suppression was previously demonstrated with 10-50 μg ssDNA/ml (Roder *et al.*, 1978b). Further attempts using more mice also failed to demonstrate the suppression and, in fact, 50 μg of ssDNA increased the anti-ssDNA response of CBA spleen cells in some instances (Table 4.2). The anti-ssDNA plaques generated were all inhibited by free ssDNA incorporated into the agarose in the assay in a dose-dependent fashion regardless of whether ssDNA was added to the cultures or not (Tables 4.1 and 4.2). These tables also show that the background response to SRBC was not affected and was not inhibited by free ssDNA in the assay as demonstrated previously in control experiments (Figure 3.1).

Since the formation of anti-ssDNA plaques is sensitive to small amounts of free ssDNA incorporated into the agarose, the suppression demonstrated by the addition of ssDNA to cultures may have actually been due to the carryover of part of this ssDNA into the assay. Because the preparation of cells from culture for plaque assay routinely involved 2 complete washes, and not just centrifugation when taken out of culture and resuspended in BSS, the effect of number of washes on plaque production was studied. It can be seen from Table 4.3 that insufficient washing of the cells is a detriment to the optimal formation of plaques. The same trend was seen whether B/W or CBA cultured spleen cells were used and was more

pronounced when ssDNA (from either of two sources) was added in culture in attempts to cause "suppression". The background anti-SRBC response was not dramatically affected by inadequate washing. The lower anti-ssDNA PFC response of inadequately washed cells was found to be due to carryover of culture material into the assay (probably DNA released from cells dying in culture) since supernatants of cells which were only washed once suppressed plaque production of cells which had been washed twice (Table 4.4, SN from 1st wash groups versus controls). Supernatants of cells which had not been washed, that is, just spun down after harvesting from culture and resuspended in BSS, suppressed plaque production even further (Table 4.4, Culture SN groups versus SN from 1st wash and control groups). Cells which had been washed twice and then given Culture SN, were subsequently spun down after samples were taken for plaquing and then resuspended in fresh BSS. Table 4.4 shows that the PFC response of these cells is beginning to approach control values again. Since the "suppression" could be removed with adequate washing the "receptor-blockade" mechanism put forward previously in the literature (Roder *et al.*, 1978b) was ruled out and is actually probably due to the carryover of DNA into the assay which can be overcome by adequate washing of the cells after harvest.

4.3.2 Effect of T Cell Depletion on Anti-ssDNA Response

In order to determine the T cell dependency of the spontaneous *in vitro* IgM-anti-ssDNA response, a comparison of the PFC responses between untreated and T-depleted CBA/J spleen cells was made. The top half of Table 4.5 shows that the spontaneous anti-ssDNA response was unaffected by T cell depletion. This is in agreement with Roder *et al.*, 1978a and Sawada and Talal, 1979, and not in total disagreement with Laskin *et al.*, 1986, who found that T cells were needed for *maximal* anti-DNA Ab production. The need for T cells for *maximal* anti-DNA Ab production was also demonstrated since the concentration of IgM anti-ssDNA

antibody in T-depleted cultures was only 56% that of non-depleted cultures (Table 4.5). If one takes into account that both cell populations were plated at the same concentration and that B cells are enriched by approximately two-fold in the T-cell-depleted cultures, the number of PFC were actually reduced by about 22% and, hence, the reduction in IgM-anti-ssDNA Ab is probably closer to 70%. Furthermore, removal of T cells reduced the amount of antibody produced on a per cell basis by 32% (Table 4.5). Profound T cell depletion was obtained in these experiments as evidenced by lack of proliferative response to Con A while the response to LPS was enhanced slightly (Table 4.5, bottom).

4.3.3 Inhibition of Plaques with Con A

The inhibition of IgM-anti-ssDNA plaques by the Con A method of Nordin *et al.*, 1969, has previously been reported (Roder *et al.*, 1978a) and was used to further characterize the nature of the plaques produced. Con A prevents fixation of complement by binding to mannose and N-acetylglucosamine end-groups (Goldstein and Iyer, 1966) present on IgM. Table 4.6 shows that, using this method, normal CBA/J mice expressed very low amounts of IgG-anti-ssDNA plaques while pre-autoimmune B/W mice had a significantly higher amount of non Con A-inhibitable plaques which was even higher (almost 100%) in old autoimmune mice.

4.3.4 Effect of RF on Anti-ssDNA Antibody Avidity and Number

To determine if blocking Fc signals had any effect on the spontaneous anti-ssDNA response, RF was added, at culture initiation, to cultures of spleen cells from four different mouse strains (CBA/J, DBA/2, Nude, B/W). Tables 4.6a, 4.6b and 4.6c, respectively, show that RF did not affect the numbers of anti-ssDNA PFCs generated, the background anti-SRBC responses or cell viabilities. However, the results of Table 4.7 demonstrate that RF can *lower* the *avidity* of the anti-ssDNA antibody in a dose-dependent fashion. This effect was more pronounced in young normals and nude mice than in old normals and B/W mice. The effect seen was not

due to the presence of RF in the assay since addition of RF to cell suspensions used for plaquing had no effect on either anti-ssDNA Ab number (Table 4.7) or avidity (Table 4.8). These observations were further supported by determination of antibody concentrations and dissociation constants by ELISA. Table 4.9 shows that addition of RF at culture initiation can decrease to some degree the level of IgM-anti-ssDNA antibody in the culture supernatant in a dose-dependent fashion. More dramatically, it results in an *increase* in the dissociation constant measured by inhibition ELISA and, therefore, a *decrease* in the IgM-anti-ssDNA Ab avidity. This effect, again, was not due to non-specific binding or measurement of the RF added since RF added directly to the assay procedure resulted in no change from control values. Addition of TRF to culture at 48 hours gave an increased IgM-anti-DNA level but also decreased the avidity. This could be interpreted to mean that the increase in K_D was due to the higher antibody level in the TRF group since all supernatants were used at the same dilution in this group of experiments. Therefore, the decreased avidity seen in the RF groups is probably underestimated since antibody levels were lower than control levels.

TABLE 4.1

Lack of Inhibition of Spontaneous Anti-ssDNA Response by ssDNA - Dose-Response Study

Group	Uninhibited Plaques		10 μg ssDNA/slide		
μgssDNA/ culture 0 hour	ssDNA	SRBC	ssDNA	SRBC	Cells recovered/ culture
CBA					
None	1745±466[a]	70±60	140±60	80±20	3.03±.03
1 μg	1460± 20	10±10	--	--	2.74±.01
10 μg	187[illegible]+438	92±97	60± 0	50±10	2.75±.29
50 μg	25[illegible] .1005	45±29	80±40	30±10	2.16±.20
100 μg	2295±827	40±20	130±10	50±30	2.03±.21
PBS 100 μl	1800±165	25±15	140±40	100±20	2.24±.20
B/W					
None	1625±175	13±12	25±25	50± 0	2.75
10 μg	1125± 50	25± 0	13±12	25± 0	3.20
50 μg	1050±100	50±50	13±12	0 ± 0	2.80
100μg	1475± 25	25± 0	13±12	0 ± 0	2.85

[a]PFC/culture ± standard deviation.

TABLE 4.2

Inability to inhibit spontaneous anti-ssDNA response by ssDNA in normal and autoimmune mice

Group	Anti-ssDNA PFC+ μg ssDNA/slide			Anti-SRBC PFC+ μg ssDNA/slide		Cells Recovered X10^6
	--	1μg	10μg	--	1μg	
CBA cells +						
-	960±458[a]	270±160	51±39	38±30	45±45	2.85±.66
50 μg ssDNA	2019±1274	790±876	50±37	60±63	--	3.23±.88
Young B/W cells +						
--	2093±1105	1400±723	28±17	129±127	132±99	3.62±.67
50 μg ssDNA	1821±825	1080±412	169±134	198±139	210±47	3.66±.66
Old B/W cells +						
--	4277±3094	3127±2394	390±30	321±243	337±120	3.71±.89
50 μg	3210±1931	1645±956	285±68	204±72	--	3.71±.95

[a]PFC/culture ± standard deviation.

TABLE 4.3

Effect of Inadequate Washing of Cultured Cells on Anti-ssDNA Response

Groups	Number of Washes: Two		One		None	
	αDNA	αSRBC	αDNA	αSRBC	αDNA	αSRBC
B/W cells +						
-	2701± 469(8)[a]	90± 19(8)	2211± 423(8)	83± 23(6)	69± 26(5)	60± 18(4)
50 μg ssDNA (Sigma)	2542± 381(7)	81± 16(6)	1524± 192(7)	75± 22(8)	19± 8(7)	23± 10(6)
CBA cells +						
-	1655± 364(4)	10± 0(2)	2000± 80(2)	5± 3(2)	5± 3(2)	5± 3(2)
50 μg ssDNA (Sigma)	1435± 102(12)	40± 6(12)	1235± 111(8)	24± 4(7)	36± 13(8)	5± 2(4)
50 μg ssDNA (P-L Biochemicals)	1205± 31(4)	90± 7(2)	540± 42(2)	0± 0(2)	30± 7(2)	10± 7(2)

[a]PFC/culture ± standard error (n).

TABLE 4.4

Suppression by ssDNA Due to Carryover of DNA into Assay

Group in assay	Cells in culture + No DNA		Cells in culture + 50 μg ssDNA	
	αDNA	αSRBC	αDNA	αSRBC
B/W Twice-washed cells +				
-	2701±469(8)[a]	90±19(8)	2542±381(7)	81±16(6)
SN from 1st wash	967±261(8)	25± 6(6)	318±145(7)	31± 9(6)
Culture SN	28± 7(5)	45± 8(4)	19± 7(7)	28± 6(6)
SN C, then resuspended in fresh BSS	1586±519(3)	80± 9(2)	907±207(4)	55±24(2)
CBA Twice-washed cells +				
-	2280±119(2)	10±0(2)	1715±163(4)	17± 7(4)
SN from 1st wash	730±150(2)	0±0(2)	205± 17(4)	5± 2(4)
Culture SN	85± 34(2)	15±4(2)	17± 6(4)	0±0(4)
SN C, then resuspended in fresh BSS			980(1)	

[a]PFC/culture ± standard error (n).

TABLE 4.5

T Cell Dependency of Anti-ssDNA Response

Group	Normal	T-Depleted	Reduction
PFC/Culture ± SE			
	1281 ± 30	1060 ±113	17%
PFC/10^6 cells recovered ± SE			
	360 ± 8[a]	563 ± 60	NA
PFC/10^6 B cells			
	720	563 ± 60	22%
Cell recovered x 10^6 ± SE			
	3.56 ± .27	1.88 ± .30	48%
[IgM-αssDNA Ab] in μg/ml supernatant ± SE			
	1.024 ± .147	0.576 ± .112	44%
IgM-αssDNA Ab/IgM-αssDNA Ab-producing cell			
	.799 ng	0.543 ng	32%
Proliferative response to:			
Medium	44,022 ± 4,236[a]	33,788 ± 1,281	
Con A	263,975 ± 5,327	35,703 ± 1,728	
LPS	223,645 ± 6,434	217,791 ± 9,600	

[a] measured as uptake of ^{3}H-thymidine in cpm/minute ± SE.

TABLE 4.6

Elimination of IgM-anti-ssDNA Plaques With Concanavalin A

Group	PFC/10^6 ± SD		
Age	No Con A	With Con A	% Non-inhibitable (IgG plaques)
CBA/J 8 wks	1063 ± 25	31 ± 22	2.9
B/W 6-10 wks	1258 ± 248	557 ± 313	44.3
B/W 10 mos	357 ± 120	335 ± 254	93.8

TABLE 4.7

Effect of RF on Anti-ssDNA Response

A. Number of Anti-ssDNA PFC/10^6 ± SE

Mouse Strain[a]	10^7 Spleen Cells + No RF	RF (1-3 μg) 0 hr	RF (10-30 μg) 0 hr
CBA/J	922 ± 35(4)	1097 ± 252(4)	940 ± 116(4)
DBA/2	222 ± 71(14)	261 ± 82(14)[b]	204 ± 60(12)
Nude	598 ± 414(4)	483 ± 222(4)	381 ± 169(4)
B/W	830 ± 188(4)	891 ± 489(4)	700 ± 152(4)

[a]For ages of mice see Table 4.8.
[b]RF (1-3 μg) added at assay: 263 ± 59 (8).

B. Number of Background Anti-SRBC PFC/10^6 ± SE

CBA/J	109 ± 122(4)	90 ± 86(4)	96 ± 94(4)
DBA/2	43 ± 22(14)	45 ± 13(14)[c]	36 ± 21(12)
Nude	20 ± 0.7(4)	24 ± 12(4)	80 ± 0(4)
B/W	86 ± 25(4)	78 ± 14(4)	105 ± 68(4)

[c]RF (1-3 μg) added at assay: 60 ± 29 (8).

C. Number of Viable Cells Recovered/Culture X 10^6 ± SE

CBA/J	4.92 ± 1.53(2)	4.92 ± 1.19(2)	4.29 ± 1.23(2)
DBA/2	2.38 ± 0.88(7)	2.07 ± 1.00(7)[d]	2.04 ± 0.74(6)
Nude	1.04 ± 0.06(2)	1.10 ± 0.25(2)	0.88 ± 0(2)
B/W	4.44 ± 0.59(2)	4.23 ± 0.89(2)	4.08 ± 0.52(2)

[d]RF (1-3 μg) added at assay: 2.08 ± 1.19 (4).

TABLE 4.8

Effect of RF on Anti-ssDNA Antibody Avidity as Determined by Plaque Inhibition

Mouse Strain[a]	10^7 Spleen Cells + No RF	10^7 Spleen Cells + RF (1-3 μg) 0 hr	10^7 Spleen Cells + RF (10-30 μg) 0 hr
CBA/J	0.053 ± 0.037[b]	0.069 ± 0.032	0.295 ± 0.135
DBA/2	0.180 ± 0.067	0.389 ± 0.099[c]	0.418 ± 0.115
Nude	0.026 ± 0.000	0.071 ± 0.087	0.250 ± 0.130
B/W	0.265 ± 0.155	0.205 ± 0.095	0.725 ± 0.376

[a]Ages of the various mice were as follows: CBA/J, 6-8 weeks; DBA/2. 4-6 months; Nude, 6-8 weeks; B/W, 8-10 weeks.
[b]Plaque avidity is expressed as the concentration of ssDNA (μg/ml) required to cause 50% inhibition of plaque formation in the assay system ± SE.
[c]RF (1-3 μg) added at assay: 0.150 ± 0.064.

TABLE 4.9

Effect of RF on K_D of Anti-ssDNA Ab from Normal DBA/2 Mice

Group	[IgMαssDNA Ab]	K_D	p-value for K_D[a]
- (control)	0.873 ± .153(12)[b]	0.070 ± .016(12)[c]	
RF 1 μg/ml	0.744 ± .129(12)	0.144 ± .022(11)	.09
RF 10 μg/ml	0.576 ± .088(4)[d]	1.261 ±1.059(3)	0.000
RF 1 μg/ml (at assay)	0.872 ± .198(8)	0.074 ± .028(8)	0.73
TRF	1.392 ± .175(8)	0.173 ± .062(8)	0.129

[a]Compared to control group.
[b]μg/ml supernatant ± SE (n).
[c]Dissociation constant x 10^{-10} $mole^{-1}$ ± SE (n).
[d]p-value = 0.05 compared to control group.

4.4 DISCUSSION

Having shown that the antibody response to a foreign antigen is regulated early by negative Fc signalling mediated by endogenously produced IgG, it was of interest to determine whether the autoimmune response was also regulated in a similar manner. If so, prevention of Fc signalling with an Fc blocking agent such as rheumatoid factor should be able to derepress a suppressed antibody response against an autoantigen. Attempts were made to inhibit the spontaneous generation of anti-ssDNA plaques *in vitro* by the addition of high molecular weight ssDNA as described previously by Roder *et al.*, (1978b). Unfortunately, this "suppression" could not be demonstrated and, furthermore, what suppression was found could be removed with adequate washing of the cultured cells. The supernatants from these washes were shown to suppress the generation of anti-ssDNA plaques while not affecting the background anti-SRBC response and it was concluded that the decrease in plaques seen with addition of ssDNA to culture was actually due to carryover of free ssDNA into the assay. This explanation is in keeping with the results (but not the conclusions) obtained by Roder *et al.*, (1978b) since they were able to show that the suppression could be achieved even when free ssDNA was added as late as 4 hours before the assay was performed. Hence, it is improbable that a "receptor blockade" mechanism was responsible since, by that time of the late addition, anti-ssDNA antibody-producing cells were at their peak. The decrease in plaque avidity reported by these authors (by plaque inhibition) would be expected as a result of free DNA carryover since high avidity plaques would be inhibited first and the remaining lower avidity plaques would require higher amounts of free DNA to be inhibited.

It was determined that the spontaneous IgM-anti-ssDNA response measured was T-independent but that maximal anti-DNA production was seen in the presence of T cells. This is in keeping with previous reports (Roder *et al.*, 1978a; Laskin *et al.*,

1986). T cells have been shown to play a major role in the regulation of the IgG-anti-DNA response, especially with regards to the switch and production of cationic IgG autoantibodies (Datta *et al.*, 1987; Sekigawa *et al.*, 1987. The requirement for T cells may not only be to facilitate the switch to IgG but also to prevent Fc signalling.

Elimination of IgM-anti-ssDNA plaques with Con A showed that non-autoimmune-prone mice had a very low number of IgG-anti-ssDNA producing plaques. The amount of non-Con A-inhibitable plaques was significantly higher in young, pre-autoimmune B/W mice (44%) and an overwhelming majority (94%) in the old, autoimmune B/W mice were of IgG isotype as was expected, and in agreement with antibody levels determined by ELISA (Chapter 3). However the high percentage (44%) of non-inhibitable plaques exhibited by the young B/W mice was not expected and is worthy of an explanation since ELISA data revealed that these mice did not have IgG-anti-ssDNA antibody levels much above that of normal mice. Con A prevents fixation of complement in the plaque assay by binding to mannosyl and N-acetylglucosaminyl ending residues found on IgM and a very small (5%) percentage of IgG immunoglobulins (Leon, 1967). The results could, in fact, be explained if B/W mice had a glycosylation defect in the addition of these end-group sugars, a defect which would correlate with the IgG-glycosylation defect reported in autoimmune diseases most notably rheumatoid arthritis (Parekh *et al.*, 1985; Rook, 1988). Further differences in glycosylation patterns have been described in the case of asymmetric, non-precipitating IgG antibodies (Margni and Binaghi, 1988) which are functionally univalent (thus, decreased avidity) due to the presence of a prosthetic carbohydrate group (N-acetylglucosamine) on one of the Fab arms of the antibody molecule (Labeta *et al.*, 1986). Cytophilicity of these antibodies via their Fc portions is maintained but effector function is lost, among them being complement fixation (Margni and Binaghi, 1972; Margni, *et al.*, 1980). This may actually be the reason for the requirement of the use of facilitating serum

for the optimal detection of IgM-anti-ssDNA plaques (Roder *et al.*, 1978a; and this thesis), something which seems paradoxical since the *in vitro* system used supports a predominantly IgM response. A higher presence of non-precipitating antibodies may be part of the dysregulation evoked *in vitro* with regards to the spontaneous anti-ssDNA antibodies. The loss of effector function of the Fc portion of non-precipitating antibodies strongly suggests that they cannot transmit negative Fc signals. This is an area that requires further investigation. Most of the data reported in the area of non-precipitating antibodies is in regards to the IgG isotype and, as yet, there are no reports available regarding other isotypes. Although these antibodies constitute about 5-10% of the normal response (Cordal and Margni, 1974), it is noteworthy that they are formed in higher amounts (as high as 65%) under conditions of prolonged immunization with particulate antigens (Margni *et al.*, 1983) and in chronic infection (Carbonetto *et al.*, 1986). This situation closely resembles the autoimmune disease state and a higher percentage of non-precipitating anti-DNA antibodies may account for the lower avidity seen in autoimmune mice (Chapter 3), especially if the negative Fc signalling mechanism is not operating. It is speculated that the form of antigen presentation modulates the ratio of non-precipitating to precipitating antibodies (Margni *et al.*, 1986).

Although suppression of the spontaneous anti-ssDNA response by the *in vitro* addition of ssDNA was not demonstrated, it was still of value to ascertain whether addition of rheumatoid factor (blockade of Fc signals) had any effect on this autoimmune response. To this end, it was demonstrated that the *in vitro* addition of RF did not affect the number of anti-ssDNA PFC generated, the number of background anti-SRBC PFC, or the cell viability. However, it was observed, in the four different mouse strains tested, that the *in vitro* addition of RF resulted in a dose-dependent *decrease* in the avidity of the anti-ssDNA antibody as determined both by plaque inhibition and competitive inhibition ELISA assays. In the case of

the normal mice, the avidity was decreased to the level of the autoimmune mice. The addition of TRF to cultures also resulted in a decrease in avidity but the antibody level was also higher in these supernatants whereas the IgM-anti-ssDNA antibody level was decreased in the presence of RF. The reason for this decrease in avidity of IgM in combination with a decrease in IgM-anti-DNA antibody level is not totally obvious when one takes into account that all supernatants were tested at the same dilution. However, the preponderance of lower avidity IgM antibodies would result if a negative regulatory mechanism needed to control the IgM to IgG switch were not operating. In the absence of this mechanism, high avidity IgM-producing cells would be more prone to switch to IgG synthesis while those of lower avidity would remain in the IgM-producing stage due to insufficient positive signals. The regulatory mechanism needed to control the switch is one which incorporates negative Fc signals since addition of RF, an Fc blocking agent, resulted in lower and not higher avidity IgM antibodies. The effect seen was not due to RF binding non-specifically to DNA, since the RF used was one which had no DNA-binding ability (Table 2.7), and furthermore, if it could, those supernatants, including those to which RF was added at the time of assay, would have demonstrated an increase in IgM-anti-ssDNA antibody levels by ELISA which they did not. Attempts were made (data not shown) to determine whether there was a concomitant increase in IgG-anti-ssDNA antibody in these cultures but it could not be demonstrated. This is not surprising since any IgG produced would be consumed and hidden by binding to the RF. However, as in the Fc signal blocking studies in responses to a foreign antigen (Sinclair and Panoskaltsis, 1987c; 1989; and Chapter 2 of this thesis), the definitive experiment is not to isolate the immunoregulatory IgG (since one could not then prove that it was regulatory in the system from which it was taken), but to prevent it from fulfilling its function by the addition of agents which block Fc signals. This is, again, indirect but more definitive evidence that immunoregulatory IgG is present in

these cultures. In fact, attempting to study the avidities of the IgG produced in normal and autoimmune mice will most likely be thwarted by factors over which one has absolutely no control, such as consumption of the antibody by immune complexes and subsequent clearing by the reticuloendothelial system which will affect the consistency and repeatability of the readout. Since addition of an agent which binds to the Fc portion of IgG results in an effect, it is only reasonable to infer that IgG is present and acting in some capacity. One could raise the argument that RF bound to high avidity IgM antibodies in the cultures and facilitated their consumption, but the specificity experiments of Chapter 2 showed that IgM could not abrogate the reconstituting effect of the RF whereas intact IgG could. Even if the above argument were true, it could not explain the lower avidity of the plaques generated from the autoimmune mice since this method looks at antibody avidity *at the time of production*.

CHAPTER 5 CONCLUSIONS AND FUTURE EXPERIMENTS

It has long been known that passive administration of antigen-IgG-antibody complexes can inactivate B cells by a mechanism which involves crosslinking of antigen receptors with Fc receptors and, hence, is dependent on the Fc portion of the IgG antibody molecule. Although much information has accumulated regarding the biochemical events which are induced as a result of engaging antigen receptors and crosslinking them with Fc receptors, the mechanisms of B cell activation and its regulation remain unresolved (unbeknownst to most). It was not until recently that evidence was provided for an Fc-dependent inactivation mechanism involving antigen and *endogenously* produced IgG antibody (Sinclair and Panoskaltsis, 1986a; 1987c). These were the first experiments for which one could correctly apply the words "negative-feedback" since studies which demonstrate suppression by passively added antibody do not demonstrate "feedback", but the potential ability for IgG antibody to be regulatory. In order to demonstrate that IgG end-product antibody regulates B cells early upon exposure to antigen, T cells had to be removed because T cell help interferes with Fc-dependent antibody-mediated suppression. It was subsequently shown that a T cell-dependent response could be mounted in the functional lack of T cell help if negative Fc signals were prevented early in the immune response by the addition of an agent which bound to the Fc portion of IgG and, hence, prevented the antigen receptors from becoming crosslinked to Fc receptors. These experiments were important for three reasons. First, they provided evidence for low levels of IgG antibody being present from the induction of the response. Second, it was demonstrated that negative Fc signals operate early and that Fc-blocking agents could replace the need for T cell mediated help. Thus, the rate-limiting role of helper T cells is to prevent Fc-mediated end-product feedback. Third, they demonstrated that if the negative signals are prevented, the B cell can respond without the need for helper Signal 2, meaning that Signal < 1 must

be a positive (and not a negative or null) stimulus. Because these early studies entailed the use of heterologous goat-anti mouse IgG-Fc antibodies in a murine *in vitro* system, it was important to demonstrate that a naturally-occurring murine Fc-binding agent could also prevent negative signals in the murine system. To this end, a set of five mouse monoclonal IgM anti-IgG-Fc antibodies, known as rheumatoid factors, were studied. It was shown that these RFs could reconstitute T cell-dependent primary and secondary antibody responses in the absence of T cell help in much the same manner as the heterologous goat antibodies. In order to reconstitute, RF had to be present from culture initiation and the ability to replace T cells could only be seen in T cell-depleted, antigen-stimulated cultures. The effect seen was specific for the Fc portion of IgG since intact, nonspecific IgG, and not $F(ab')_2$ or IgM, could abrogate the ability of RF to reconstitute the response. Not all RFs were equal in their ability to substitute for T cell help. One of the five RFs, RF4 (IgM-anti-IgG_{2a}) exhibited superior reconstituting capabilities and was used in the majority of the experiments reported. This RF did not demonstrate any occult stimulatory ability other than as an Fc blocking agent since it could not reconstitute when added at 2 days of culture and, furthermore, it did not demonstrate IL-4 activity which has been shown to interfere with Fc signals. The superior reconstituting abilities of RF4 may be due to a higher affinity for that portion of the Fc of IgG which is responsible for emission of the negative signal. Since carbohydrate is necessary for IgG to be immunosuppressive, it would be interesting to find out whether RFs with a higher affinity for Fc in the context of carbohydrate moieties are those which can prevent negative Fc signals. For this to be accomplished, a large panel of monoclonal RFs well-characterized with regards to their specificity should be studied.

It is important to note that the negative signalling model predicts that IgG RFs (as opposed to the IgM RFs used) with high affinity for Fc will be highly

immunosuppressive. Although negative signals, emitted from the Fc portion of IgG against the stimulating antigen, will be prevented, the antigen receptor-Fc receptor crosslink will still be achieved but via the Fc portion of the IgG RF. There is evidence that graft survival correlates with the presence of IgG RF (Terness *et al.*, 1989; Susal *et al.*, in press) and the isolated IgG RF was shown to be immunosuppressive in an Fc-dependent fashion. In order to demonstrate that this IgG RF is indeed immunoregulatory, the obvious experiment to be performed is to add non-immunosuppressive IgM RF to prevent the emission of negative Fc signals from the IgG RF and monitor the graft outcome.

The *in vitro* spontaneous anti-ssDNA response was studied to provide insights into the regulation of the autoimmune response and aberrations thereof which give rise to clinical autoimmunity. It was demonstrated that normal non-autoimmune-prone mice had *higher* avidity IgM-anti-ssDNA antibodies than the autoimmune-prone New Zealand mice, contrary to what would have been expected. Since high avidity antibody is a more efficient immunosuppressant, the results provide evidence that, in non-autoimmune-prone animals, autoreactive B cells are down-regulated by low levels of high avidity IgG antibody and the progression through the IgM to IgG switch would be prevented. Thus, the dysregulation exhibited in the *in vitro* system used would allow the activity of these high avidity autoreactive cells. Since B cell Fc receptors are incompetent in the NZ mice (Uher and Dickler, 1986b), Fc feedback would not be efficient and autoreactive cells would be allowed *in vivo* to switch to IgG autoantibody production and effect tissue damage. Using transgenic experiments, then, this model would predict that insertion of a defective B cell Fc receptor into normal mice should allow the emergence of autoimmune disease. The converse (i.e. insertion of a functional Fc receptor to prevent disease in autoimmune-prone mice) may not be viable since autoimmune disease is so fraught with other factors that can interfere with effective negative Fc signalling such as the

glycosylation defects of the Fc portion of IgG and an abundance of helper T cell activity. In addition, comparisons between normal and autoimmune-prone mice for avidities of antibodies against other autoantigens (histones, myosin, actin, thyroglobulin, etc.) should also be done.

There have been recent reports that the anti-DNA response can be suppressed by antibodies specific for DNA (Hahn and Ebling, 1983) or the predominant anti-DNA idiotype (anti-id Abs) (Abdou *et al.*, 1981; Hahn and Ebling, 1984;), while others (Teitelbaum *et al.*, 1984) have, to the contrary, demonstrated an increase in anti-DNA antibody production. Other reports have failed to demonstrate the presence of anti-idiotype following autoantibody seroconversion (from positive to negative) (Kyner *et al.*, 1989). Notwithstanding this, it would be important to define the mechanism of this suppression, or failure thereof, by anti-idiotypes (Kearney and Vakil, 1986). Is this an Fc-dependent mechanism? If not apparently so, Fc signals by endogenous IgG coopted by the anti-id would have to be ruled out by the addition of Fc blocking agents. Other investigations on the effects of passively administered IgG-autoantigen (DNA) complexes on the anti-DNA response, and whether suppression is Fc-dependent, should also be done.

The effect of RF on the regulation of an autoimmune response was also investigated. It was demonstrated that RF had no effect on the spontaneous *in vitro* anti-ssDNA response with regards to number of PFC generated, contrary to the increase that was expected in view of the reconstitution experiments of the anti-SRBC response. However, RF was able to *decrease* the avidity of the IgM-anti-ssDNA antibody formed *in vitro*. This is in keeping with negative Fc signalling by endogenous IgG playing a role in the regulation of the IgM to IgG switch as outlined above.

It is tempting to speculate that high levels of RF should correlate with low avidity IgM antibodies and the IgM to IgG switch. This may not be necessarily so

since RF levels have been found to be not so useful as a diagnostic tool. These studies should not be taken at face value to mean that RF always serves to block Fc signals or that high levels of RF are necessary to cause aberrant negative Fc signalling since not all RFs used in this thesis were equal in their reconstituting capacities and not all RFs need serve the same purpose since some are just "chance" crossreactivities. Rather, these studies show that RF is a potential immunoregulator just as IgG was shown to be in the early studies on antibody-mediated immunosuppression.

Since the clonal deletion theory for the avoidance of autoimmunity states that autoreactive lymphocytes are deleted or made stably anergic during *early* ontogeny (Burnet, 1957; Nossal, 1986; 1988), a demonstration of the presence of high avidity specificities directed towards autoantigens in the later stages of lymphocyte maturation and activation by self antigens in animals, not prone to autoimmune disease, is a negation of the clonal deletion theory for self-nonself discrimination. To state this argument positively, the major question, with regard to self-nonself discrimination, is, "*At what point* in the differentiation of stem cells into B cells, or activation of B cells by autoantigen towards antibody forming cells, is self-nonself discrimination made?". It is difficult to imagine that B cells via their antigen receptors can distinguish self ligands from non-self ligands. Rather, it must be the net effect of the culmination of signals (positive and negative) received by the B cell which "decides" whether it will remain activated. The quantity and quality of these signals is, in large part, dependent on the microenvironment in which the antigen in question is presented and in what context (Sinclair and Panoskaltsis, 1988). Foreign antigen is trapped and retained by reticular dendritic cells in germinal centres and in T-dependent areas of lymphatic tissue (Klaus *et al.*, 1980; Szakal *et al.*, 1989). Antibody production takes place in the medullary region where the antigen is removed by medullary sinus macrophages. Reticular dendritic cells possess many Fc

receptors and. therefore, antigen can be presented and stimulatory to the B cell while Fcs are kept away from the B cell surface. Upon migration through the T-dependent area, B cells receive T helper signals which reduce their sensitivity to the negative influences of antigen-antibody complexes on their surface. Antibody production can then take place when antigen is removed in the medulla since the inactivating complexes are disrupted. This antigen clearance has been termed "antigen-depresentation" (Sinclair and Panoskaltsis, 1988). Because self-antigen is in continued supply, it cannot be depresented sufficiently to allow full scale antibody production. In the absence of sufficient T cell help in the T-dependent regions, RF can act as an alternative to reduce sensitivity to negative Fc signals. Tolerance to self could be achieved in the absence of autoreactive T cells (Rose *et al.*, 1981), but evidence is mounting that these cells do exist in the normal immunocompetent host (Lider *et al.*, 1986; Esquivel *et al.*, 1977; 1978; ElRehewy *et al.*, 1981). These T cells recognize an epitope composed of the self-peptide-MHC antigen complex (reviewed by Ada and Rose, 1988; Rose, 1989). Interestingly, it has been proposed that T cells with high avidity for self-antigen-MHC complexes are negatively selected in the thymus (Ada and Rose, 1988). This can be interpreted as a negative feedback mechanism much like the one reported here in which high avidity B cells are down-regulated by end-product. Low-avidity T cells which migrate to the periphery, can be activated if positive signalling reaches a critical threshold level (Rose, 1989), due to aberrant high expression of MHC II or antigen presentation by all too eager B cells. This type of B cell hyperactivity may be linked to defective regulation by Fc signals.

In conclusion, rheumatoid factor was used as an Fc signal blocking agent and shown to replace the need for T cells in the primary and secondary anti-SRBC antibody response. This was a further demonstration of the presence of immunoregulatory IgG early in the immune response. Furthermore, RF also

decreased the avidity of spontaneously formed IgM-anti-ssDNA autoantibodies which were found to be of higher avidity in normal than in autoimmune-prone mice. Fc signalling may also be important in the IgM to IgG class switch since low avidity IgM but higher levels of IgG-anti-ssDNA antibodies predominate in autoimmune mice. It would be a simple matter if one could incriminate high levels of RF as the cause of autoimmunity. However, the diathesis for autoimmunity has many etiologies. The results of this thesis, and in keeping with the poor correlation between RF levels and disease activity, demonstrate that *inefficient negative Fc signalling* has profound influences on the immune response and whether it be due to high levels of Fc blocking agents (such as RF), incompetent Fc receptors, defective negative signal delivery by IgG (due to either low avidity or defective Fc portions) or presentation of antigen in a manner which is not conducive for negative signals to be delivered, is not the issue. All these causes are not mutually exclusive. The crux of the matter is that B cells, no matter what their specificity, can be regulated by Fc signals through negative end-product feedback and this must be kept in mind in order to understand processes which lead to either immune regulation or dysregulation.

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