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A REASSESSMENT OF THE T-DEPENDENCE/INDEPENDENCE OF POLYSACCHARIDE AND PROTEIN ANTIGENS

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SUMMARY

This article reviews the literature concerning thymus independent antigens as well as presenting experimental results concerning immune responses to these antigens and their analogues. The review focuses on the nature of thymus independent antigens, the sites or tissues responsible for responses to these antigens, subpopulations of B cells responding to these and other antigens, memory to these antigens and the roles of T cell and macrophage derived factors in responses to these antigens. In the literature, there is evidence that T cells, macrophages and products from these cell types can regulate responses to so-called thymus independent antigens. It is even possible that such factors may be required for responses to these antigens.

Experiments with murine peritoneal and splenic B cells revealed that interleukin-5 can increase antibody responses to thymus independent type 1 antigens. Proliferation by murine splenic B cells stimulated with the thymus independent type 2 antigen analogue system, anti-IgD-dextran was increased by interleukin-5 as well as by IL-1. Proliferation induced by a different thymus independent type 2 analogue system using anti-IgM + dextran sulphate was increased by interleukins 4 and 5. Proliferation by mouse splenic B cells was also increased by interleukins 4 and 5 when the thymus independent antigen analogue system anti-IgM + lipopolysaccharide was used. Hence, different interleukins appear to be important for responses to different types of thymus independent antigens.

Human peripheral blood B lymphocytes proliferated in response to two thymus independent type 2 analogues, anti-IgD-dextran and anti-IgM-dextran. Proliferation by these cells was increased by interleukin-2 but not by the other interleukins tested. No evidence of B cell responses was obtained from cells prepared from peritoneal dialysis fluids of patients undergoing continuous ambulatory peritoneal dialysis. This was due to a lack of B cells in these fluids and not to immunoincompetence since a vigorous T cell response to a bacterial superantigen was observed by cells from these fluids.

Hence, various strategies for antibacterial immunity have been developed in different tissues of different species and interleukins can influence the antibody responses to some of these bacterial components.

INTRODUCTION

Protection against pathogenic bacteria is certainly one of the most important functions of an immune system and therefore it is not surprising that many

anti-bacterial strategies have been developed. Antibodies to bacteria are one of the immune systems' central defence mechanisms and they are effective in several ways including opsonisation and complement mediated lysis. Many germline antibody genes encode specificities directed against polysaccharide components among which are those found on the outer surfaces and capsules of bacteria. Interestingly, and probably not fortuitously, many of these outer components of bacteria, the polysaccharides, lipoproteins and lipopolysaccharides, can activate B lymphocytes in the apparent absence of T cells. Hence these types of antigens have been called thymus independent (TI).

TI antigens have several interesting features as do the responses to these antigens. The antigens themselves are generally polymeric in nature and some act as polyclonal activators at high concentrations. Immunological responses to these TI antigens differ from those to thymus dependent antigens in several important respects. One difference lies in the relative amounts of isotypes and subclasses of immunoglobulins produced. The secondary, or memory, responses are also somewhat different. It is now becoming clear that certain specific sites and cell types are very important in responses to TI antigens. There is also some controversy as to whether the B cells which respond to TI antigens are from the same population as those which respond to thymus dependent antigens. Finally, although responses to some of these antigens are termed thymus independent, recent findings indicate a role for T cells as well as macrophages in both generating and regulating antibody responses to these agents.

This report will deal with these issues first by reviewing some of what is available in the literature. We then present experimental findings of our own which bear on some of these issues and extend our understanding of some aspects of responses to TI antigens by peritoneal lymphoid elements and other peripheral tissues.

The nature of TI antigens and responses to them

The observation that athymic nude mice could mount an antibody response to certain antigens probably gave rise to the name thymus independent. This observation was clarified by demonstrations that purified B cells from mice (*Snow* et al., 1983), humans (*Golding* et al., 1981) and other species can make antibodies *in vitro* to so-called TI antigens. Responses to these antigens are predominantly of the IgM isotype, although IgG subclasses (*Hurme*, 1976; *Klaus* et al., 1976; *Slack* et al., 1980) as well as IgA (*Taubman* et al., 1986) can sometimes be seen. The IgM response to these antigens can be long-lived, presumably due to antigen persistence or to polyclonal activation or both.

CBA/N mice, and some other *xid* (X chromosome linked immunodeficient) mice, can respond to some TI antigens and not to others (*Mosier* et al., 1976; *Cohen* et al., 1976). This led to a classification of TI antigens into those to which CBA/N mice could respond, called TI-1 antigens, and those to which CBA/N mice could not respond, dubbed TI-2 antigens. Using the responsiveness of CBA/N mice as a criterion for TI-1 or TI-2 classification is perhaps not the best, however, since *xid* B cells can not only bind and present what have been called TI-2 antigens (*Kirkland* et al., 1980) but they can also respond to TI-2 antigens if IL-2 (*Delovitch* et al., 1983), a helper T cell population (*Braley-Mullen*, 1982; *Lindsten and Andersson*, 1979; *Klaus* et al., 1976), or a TI-1 antigen (*Couderc* et al., 1984) is concomitantly present.

Several other parameters distinguish the responses to different TI antigens, and they seem to follow a pattern. Besides the physical differences between so-called TI-1 and TI-2 antigens, considered below, the IgG subclasses evoked by these classes of antigens tend to be different. Thus while IgG_1 predominates over IgG_2 and IgG_3 in thymus dependent responses, little thymus independent IgG_1 is seen. Responses to TI-1 antigens tend to evoke comparable levels of IgG_2 and IgG_3 whereas TI-1 antigens evoke far more IgG_3 than IgG_2 (*Slack* et al., 1980).

Some TI-2 antigens tend to persist for long periods *in vivo* since macrophages apparently are ineffective at degrading them. Hence, responses to TI-2 antigens can persist for extended periods.

Another difference between responses to TI-1 and TI-2 antigens is that responses to TI-2 antigens tend to arise later in ontogeny than those to TI-1 antigens. This is true for both mice and humans (*McKearn* and *Quintans*, 1979; *Golding* and *Rittenberg*, 1984; *van Rees* et al., 1987).

Both TI-1 and TI-2 antigens cause B cell membrane depolarisation but only TI-1 antigens lead to the G_0 to G_1 transition (*Monroe* and *Cambier*, 1988). Moreover, TI-1 antigens can initiate proliferation in antigen specific B cells while TI-2 antigens appear to require interleukins, as well, for induction of proliferation (*Stein* et al., 1986).

Many of the differences in responses to TI-1 and TI-2 antigens probably result from differences in their structures. As previously stated, TI antigens tend to be polymeric. Some examples are polysaccharides like levan, dextran or ficoll and polymerised flagellin. These are considered TI-2 type antigens. Lipopolysaccharides and lipoproteins are other TI antigen examples, although these are considered TI-1 type. A controversy early in the study of TI responses centred around a putative requirement for a mitogenic carrier moiety. Early studies suggested that both TI-1 and TI-2 antigens induced B cell proliferation and the carriers needed only to be polymeric, not mitogenic or immunogenic (*Klaus* et al., 1975). The epitope density was shown to affect the immunogenicity of certain TI-2 antigens (*Desaymard* and *Howard*, 1975a; 1975b). In addition, the chain length has been implicated in the immunogenicity of TI antigens (*Peacock* et al., 1983). Several studies state that a mitogenic principle is required for responses to TI-1 antigens (*Coutinho* and *Gronowicz*, 1975; *Hare* et al., 1980; *Alarcon-Riquelme* and *Moller*, 1990). There is some confusion in the literature concerning the relative importance of each of these structural parameters contributing to the thymic independence of these antigens.

A clarifying series of experiments showed that if a polyclonal activator was present on an antigen, then a polymeric structure was not required for thymic independence of the antibody response (*Ishizaka* et al., 1979). Along these lines, TNP-liposomes have been classified as TI-2 antigens whereas insertion of lipid A (a polyclonal activator) into TNP-liposomes converts them to TI-1 type antigens (*Tadakuma* et al., 1982). This is probably a useful unifying concept: polyclonally activating moieties can render oligovalent antigens thymic independent; otherwise extensive B cell surface Ig crosslinking provided by high epitope density on repeating structures appears necessary to render responses to antigens independent of certain T cell requirements. The absence of a polyclonal activator on (TI-2) antigens probably makes them less immunogenic regardless of their polymeric nature and the extent of epitope density. This, in turn, probably explains why

responses to TI-2 antigens are more difficult to elicit and seem to have more requirements than TI-1 antigen responses.

Sites of Responses to TI Antigens

Antibody responses to different TI antigens *in vivo* reveal some differences in their induction requirements as well as differences in the characteristics of the response patterns. The locations of responses to different antigens can be different as can the cells required for, or contributing to, these antibody responses.

The spleen is a very important site of antibody formation especially in response to TI antigens. Splenectomy in rats (*Gray* et al., 1985; *Amlot* et al., 1985) and humans (*Amlot* and *Hayes*, 1985) abolishes primary *in vivo* responses to TI-2 antigens confirming that the spleen, almost exclusively, accounts for TI-2 antibody responses (*Veerman* and *Vries*, 1976; *van Rees* et al., 1987). Antibody Forming Cells (AFC's) elicited by both thymus dependent (TD) and TI antigens are first seen in the outer part of the peri-arteriolar lymphoid sheath (*Veerman* and *Vries*, 1976; *Eikelenboom* et al., 1982). Both types of antigen cause AFC localisation in the marginal zone (MZ) while there is some subsequent migration to the follicle (*Veerman* and *Vries*, 1976). Interestingly, spleens respond faster to TI antigens although most antigens must first transit through lymph nodes (*Delemarre* et al., 1989). In fact, two days after immunisation with a TI-2 antigen, AFC's can be seen in the spleen (*Claassen* et al., 1986).

Some TI-2 antigens are taken up exclusively by splenic marginal zone macrophages (*Chao* and *MacPherson*, 1990, *Humphrey* and *Grennan*, 1981). This would appear to account for the importance of the splenic marginal zone in TI-2 antigen responses. Others have shown, however, that MZ macrophages and AFC's can distribute differentially suggesting that MZ macrophages are not absolutely required for TI-2 responses. Although the requirement for MZ macrophages may not be absolute, recent reports indicated that the MZ is essential for TI-2 responses (*Claassen* et al., 1989). These observations are consistent with the report that recirculating B cells go to the splenic MZ to make TI-2 responses (*Lane* et al., 1986) whereas some other TI antigens can generate AFC's in the bone marrow (*Koch* et al., 1982) or some lymph nodes (*Goud* et al., 1990) as well as the spleen.

These and several other pieces of evidence support the concept that macrophages are required for TI-2 antigen responses. *In vivo* depletion of macrophages abolishes TI-2 antibody responses (*Delemarre* et al., 1990). The beige mouse, in addition to other immune defects, has a macrophage defect as well as impaired TI-2 responses (*Pflumio* et al., 1990). IL-1, a macrophage product, is reported to be absolutely required for TI-2 responses (*Sinha* et al., 1987). Furthermore, *in vitro* proliferation induced by fluoresceinated ficoll conjugates has been reported to require macrophages (*Pillai* and *Scott*, 1981). Marginal zone macrophages have been described as exclusively responsible for TI-2 antigen uptake (*Chao* and *MacPherson*, 1990). Together, these observations suggest that macrophages are required for TI-2 responses and a population of marginal zone macrophages alone may be competent to provide this helper function.

Subpopulations of B Cells Responding to TD, TI-1 and TI-2 Antigens

The subject of B cell subpopulations is a controversial one and this contro-

versy includes the possibility of different subpopulations responding to different antigenic forms. One early datum contributing to the idea of different subpopulations for different antigens concerns the *xid* B cell defect. Since *xid* B cells respond to TD and TI-1 antigens, it appeared that a TI-2 antigen responsive population bearing the lyb5 surface antigen was missing (*Mosier* et al., 1976, *Cohen* et al., 1976). The previously mentioned reports that *xid* B cells could respond to TI-2 antigens when a T helper cell (*Braley-Mullen*, 1982, *Lindsten* and *Andersson*, 1979, *Klaus* et al., 1976), IL-2 (*Delovitch* et al., 1983) or a TI-1 antigen (*Couderc* et al., 1984) was present confuse the issue of differentially responsive B cells subpopulations. Furthermore, neonatal CBA/N B cells respond to TI-2 antigens in the presence of T helper cells exactly as do normal neonatal CBA/J B cells in the presence of T helper cells do (*Lindsten* et al., 1979). Hence, the defect in these B cells has been described as one of development, rather than as one of a missing population. Moreover, CBA/N B cells can bind and present TI-2 antigens (*Kirkland* et al., 1980). Therefore, it is unclear how the *xid* B cell defect defines separate subpopulations of TI responsive B cells.

One report showed that anti-Lyb7 blocked TI-2 responses and not those to TI-1 antigens (*Subbarao* et al., 1979). Similarly, anti-IgM or anti-IgD will both block TD responses but anti-IgM alone blocks TI responses (*Cambier* et al., 1978). Others have shown that TI-2 responsive B cells are more radiosensitive than TI-1 responsive B cells (*Lee* and *Woodland*, 1985). These differences may simply reflect differing triggering requirements for TI-1 antigens compared to TI-2 antigens rather than the existence of separately responsive subpopulations. Indeed, by elimination experiments, it has been found that the populations responding to TD and TI antigens overlap appreciably (*Hurwitz* et al., 1982). This is probably the strongest finding and before one accepts the existence of separately responsive populations more support is necessary.

Some studies have shown that TI-2 responsive B cells are long-lived and migrate into the bone marrow (*Koch* et al., 1982). More recent studies using adoptive transfers have shown that B cells responding to TI-2 antigens are long-lived whereas TD antigen responsive B cells are both long-lived and short-lived (*Udhayakumar* et al., 1988).

It has also been shown that once B cells have been primed by a TD antigen, they can be activated by either TD or TI forms of antigen (*Rennick* et al., 1983). Furthermore, one report states that memory to both TD and TI antigens resides in the surface Complement Receptor (presumably the CR2) negative B cell subpopulation (*Lindsten* et al., 1985). Another group, however, opposes this view claiming that primed B cells fall into two distinct populations responding differentially to either TI or TD antigens (*Rittenberg* and *Tittle*, 1978).

It appears that there is no consensus yet as to whether truly distinct B cell populations responding in a primary or secondary response to TD, TI-1 or TI-2 antigens exist. Until some clarifying experiments unite all these data, caution in accepting separately responding subpopulations is probably well advised.

Generation of Memory by TI-Antigens

Although a classical anamnestic response is not usually elicited by a secondary challenge with TI antigens, there is evidence that a secondary response does occur. Some have reported a secondary response to TI-2 antigens (*Hurme*, 1976) and shown TI antigen-mediated induction of T helper cells

(*Bretscher*, 1984). Indeed, immunisation with a TI antigen yields protection against a fatal secondary challenge with *Trichinella spiralis* (*Lim* and *Choy*, 1990). Others, however, failed to observe a memory response and showed that the failure was due to suppressive anti-carrier (*Fernandez* and *Moller*, 1978) or anti-hapten (*Brodeur* and *Wortis*, 1980) antibodies. Furthermore, it has been reported that TI-2 antigens may induce suppressor T cells under some conditions (*Fraser* and *Braley-Mullen*, 1981). Indeed, nude mice, which should lack T suppressor cells, can give rise to secondary TI-2 antibody responses (*Schott* and *Merchant*, 1979).

A major factor contributing to these apparently contradictory findings concerning the induction of TI memory may be genetic. There are several reports of strain differences in the ability of TI antigens to generate memory and a secondary response (*Fernandez* and *Moller*, 1979, *Motta* and *Truffa-Bachi*, 1980, *Motta* et al., 1981, *Colle* et al., 1983, *Shidani* et al., 1983). The genetic basis for this difference is poorly understood at present, but it may relate to some triggering mechanisms. Two groups suggest that both TI-1 and TI-2 antigens can generate memory B cells but only TI-1 antigens can activate these memory cells (*Colle* et al., 1983b, *Truffa-Bachi* et al., 1983). Others claim that both types of antigen can generate memory but that lipopolysaccharide (LPS) is concomitantly required for TI-2 antigen memory generation (*Zhang* et al., 1988).

There is also some controversy concerning the cells responsible for these anamnestic responses. One group identified surface Complement Receptor-negative B cells as the carriers of memory for both TD and TI antigens (*Lindsten* et al., 1985). However, others report that the precursors for a TI-2 response are long-lived (*Udhayakumar* et al., 1988) whereas memory to TI-1 antigens declines and its maintenance requires antigen persistence and B cell renewal from the bone marrow (*Colle* et al., 1988, *Burlen* et al., 1988). These apparently differing conclusions may, in fact, not be mutually exclusive. Rather, they may simply reflect different apparent phenotypes of B cells at different stages in their response to antigen.

Role of T cell and Macrophage Derived Factors in TI Responses

Although responses to the antigens we have been considering are called thymus independent, there is much evidence to show that T cells and their products can influence these responses. For this reason, it has been proposed that these antigens be renamed thymus regulated instead of thymus independent (*Mond* and *Brunswick*, 1987). The term thymus independent originated from the observation of responses in athymic nude mice. However, we now appreciate that nude mice are seldom devoid of all T cells. Furthermore, some mediators secreted by T cells are also secreted by mast cells and large granular lymphocytes which are also present in nude mice. Hence, responses to some of these antigens may indeed be dependent on influences from T cells. Nevertheless, the term thymus independent is historical, and as such, will probably remain. It is this T cell regulation which is considered here.

Quite early it was observed that supernatants from concanavalin A (Con A) stimulated cultures (*Chen* and *Leon*, 1976) could increase responses to TI-2 antigens and that gamma interferon (IFN-γ) could suppress these responses (*Johnson* et al., 1975). These observations are consistent with the findings that optimal TI-2 induced IgG is seen in the presence of T helper cells (*Klaus* et al., 1976) and that TI-2 antigens can in-

Table 1: Interleukin-5 enhances antibody secretion by peritoneal mouse B lymphocytes induced with bacterial products

Addition	Anti-IL-5R mAb	Anti-TNP PFC/culture		
		None	DXS	LPS + DXS
Medium	-	1 ± 1	13 ± 6	373 ± 50
	+	1 ± 1	0 ± 0	400 ± 25
IL-5	-	9 ± 2	96 ± 19	1211 ± 84
	+	2 ± 2	5 ± 3	389 ± 54

5,000 Ly^+ peritoneal B cells were cultured 5 days with the indicated stimuli. DXS was used at 5 μg/ml and the monoclonal anti-IL-5R antibody R52.120 at 1 μg/ml. Standard errors for triplicate determinations are shown.

duce helper T cells (*Bretscher*, 1984). Non specific helper T cells have been shown to increase TI-2 antigen responses (*Wood* et al., 1982). In fact, T cells generally have been found to increase and suppress TI-1 antigen responses (*Tanay* and *Strober*, 1985). Furthermore, addition of Con A activated T cells can regulate TI responses (*Primi* et al., 1982) and addition of Con A itself can increase responses to TI-1 antigens (*Golding* et al., 1982). More precisely, addition of Con A to human B cells responding to the TI-1 antigen TNP-*Brucella abortus* results in recruitment of more B cell precursors and in larger B cell burst sizes (*Golding* and *Rittenberg*, 1984).

Further support for a role of T cell derived factors influencing TI antigen responses exists. Surface IgD positive neonatal B cells can respond to the TI-1 antigen TNP-*Brucella abortus* but a different set of interleukins is required to support sIgD negative B cell responses to this antigen (*McFadden* and *Vitetta*, 1984, *Waldschmidt* et al., 1985). Allogeneic amplifier T cells reportedly increase responses to TI-2 antigens but not to TI-1 antigens (*Braley-Mullen*, 1982). Cyclosporin A blocks interleukin production and secretion by T cells and also blocks secondary TI-1 antigen responses (*Shidani* et al., 1983). Similarly, *xid* B cells can respond to TI-2 antigens in the presence of IL-2 (*Delovitch* et al., 1983). These observations suggest that T cell derived interleukins can indeed be very important in responses to TI antigens.

Table 2: Interleukin-5 enhancement of antibody secretion by murine peritoneal B lymphocytes induced with the TI-1 antigen TNP-LPS

Stimulus	Anti-TNP PFC/culture		
	Medium	IL-4	IL-5
Medium	10 ± 4	56 ± 4	768 ± 60
TNP-LPS	108 ± 16	114 ± 8	1448 ± 122

50,000 murine 1.075 g/cc Percoll separated peritoneal B cells were cultured 5 days and PFC determinations were performed. Interleukins were used at 10 U/ml and TNP-LPS at 0.1 μg/ml. Standard deviations are given for triplicate determinations.

Table 3: Interleukin-5 enhances murine peritoneal B cell proliferation induced by bacterial products

Addition	cpm/culture x 10^{-3}			% of input B cells yielding clones
	Medium	DXS	LPS	LPS + DXS
none	0.5	4.9	20.4	16.8 ± 5.1
IL-2	1.2	7.5	21.9	21.1 ± 6.0
IL-3	1.3	11.4	23.7	ND
IL-4	1.8	9.3	25.1	18.3 ± 5.5
IL-5	5.2	25.9	49.8	45.7 ± 10.2

For thymidine incorporation (cpm/culture), 5 x 10^4 peritoneal B cells were cultured 2 days and pulsed during the terminal 6 hours of culture. LPS was used at 50 μg/ml, DXS at 5 μg/ml and the interleukins at 10 U/ml. For determination of the percentages of responding cells, Poisson analyses were performed on peritoneal B cells cultured at about 1, 2, 4 and 8 cells/well for four days. Each input contained 60 microcultures. Frequencies are presented with associated 95% confidence intervals.

More precise studies, using purified and cloned interleukins have successfully demonstrated their roles in TI antibody responses. Several groups have shown that IL-1 helps TI responses (*Pike* et al., 1987; *Stein* et al., 1986; *Wetzel*, 1989; 1990) and some even suggest that IL-1 is absolutely required for responses to both TI-1 and TI-2 antigens (*Sinha* et al., 1987). In contradistinction to some (*Pike* et al., 1987), a role for IL-4 in TI-2 antigen responses has been proposed (*Stein* et al., 1986). These same investigators, however, assert no relevant role for IL-2 or IL-5 (*Stein* et al., 1986). This is in contrast to other studies which do demonstrate IL-2 (*Pike* et al., 1987, *Mond* and *Brunswick*, 1987, see below) and IL-5 (*Pike* et al., 1987, *Wetzel*, 1989, 1990, 1991a, see below) involvement in responses to TI antigens.

The weight of the evidence suggests that interleukins can not only regulate TI antibody responses, but may in fact be required in some cases. The experimental sections which follow address the specific contributions of some interleukins, notably IL-1, IL-2, IL-4 and IL-5 in responses to TI antigens and analogues to try to extend our understanding in this area.

MATERIALS AND METHODS

Murine Studies

All the materials and methods for the experiments with peritoneal and splenic mouse B lymphocytes have been described previously (*Wetzel*, 1991a,b, 1990, 1989).

Human Studies

Cells:

Human peripheral blood lymphocytes were obtained by separating buffy coats from normal donors on Lymphocyte Separation Medium followed by treatment with 1 mM L-leucyl methyl ester for 30 minutes at room temperature. T cells were then depleted by rosette formation with AET treated SRBC and subsequent centrifugation on Lymphocyte Separation Medium. In some cases T cells were further depleted by treatment with anti-CD8 monoclonal antibody plus complement. Peritoneal dialysates were obtained by informed consenting patients undergoing continuous ambulatory peritoneal dialysis as outpatients at the George Washington

Table 4: Comparison of the ability of different interleukins to enhance antibody secretion by splenic murine B cells induced with the TI-1 type antigen TNP-LPS

Additions	anti-TNP PCF/10^6 cells (x 10^{-3})
none	0.8 ± 0.1
rIL-2	0.6 ± 0.1
rIL-4	0.8 ± 0.1
rIL-5	2.5 ± 0.5

100,000 resting murine splenic B cells were cultured 5 days with 0.1 μg/ml TNP-LPS. The indicated interleukins were used at 10 U/ml. Standard deviations are given. Shown are the results of three experiments.

Medical Center at the George Washington University in Washington DC and were obtained and provided kindly by Dr. Susan Lu. Cells were obtained by centrifugation and were treated with L-leucyl methyl ester as described above.

Reagents:

The monoclonal anti-IgD-dextran and anti-IgM-dextran conjugates were prepared by Dr. Andrew Lees and were provided as a kind gift. PHA-P and staphylococcal enterotoxin B were purchased from Sigma, St. Louis, MO. The human interleukins, with the exception of recombinant human IL-5 which was prepared by Dr. Tavernier from Hoffmann La Roche and was provided as a gift, were purchased as recombinant materials from Boehringer Mannheim and Genzyme.

RESULTS

The peritoneum of mice is a location where one might expect encounter with bacterial products. Hence we examined the antibody response of purified murine peritoneal B lymphocytes to two polyclonal activators which are, or mimic, bacterial products: lipopolysaccharide (LPS) and dextran sulphate (DXS). It can be seen in Table 1 that DXS by itself induced a weak Plaque Forming Cell (PFC) response which was increased by interleukin-5 (IL-5). LPS+DXS induced a much larger response which could be increased about threefold by IL-5. This IL-5 mediated increase was blocked by monoclonal antibody to the IL-5 receptor.

Next, the response of these peritoneal B cells to a prototype TI-1 antigen, TNP-LPS, was examined. The influence of two different interleukins on the antibody response was observed and these are shown in Table 2. TNP-LPS by itself was able to stimulate a vigorous PFC response at both antigenic and polyclonally activating doses. Among the interleukins tested, IL-5 alone increased the observed levels of PFCs.

The ability of these bacterial products to stimulate proliferation in these peritoneal cells was then examined. Results in Table 3 measure both thymidine incorporation and the fraction (%) of responding B cells. These measurements showed that, of the tested interleukins, IL-5 was the most effective at enhancing B cell proliferation stimulated by LPS+DXS. In the presence of IL-5, two- to three- fold more B cells were recruited to proliferate than observed with the combination of LPS+DXS alone.

The peritoneal B cells examined in

Table 5: The effect of different interleukins on resting murine splenic B cell proliferation induced by the thymus independent antigen type 2 analogue anti-∂-dextran

	cpm/culture x 10^{-3}							
	Addition							
Anti-∂-dextran (ng/ml)	None	IL-1	IL-2	IL-3	IL-4	IL-5	IL-6	IFN-γ
0.0	2.8	3.8	3.2	1.9	3.0	6.0	2.0	2.3
0.01	2.9	4.9	2.7	2.5	4.6	9.1	2.7	4.0
0.1	20.6	27.7	14.3	12.8	23.5	34.3	15.9	15.7
1.0	135.6	140.7	95.3	102.6	94.2	161.4	71.2	58.2

10^5 resting murine splenic B cells were cultured 2 days and pulsed with tritiated thymidine during the terminal 6 hours of culture. 100 U/ml IL-1, IL-6 and IFN-γ were used whereas 10 U/ml of the other interleukins were used.

the first three tables were larger and more metabolically active that their resting B cell counterparts from the spleen or other sites. Since there is some traffic between the spleen and peritoneum, and since splenic precursors can participate in antibody responses at other locations, we decided to investigate the response characteristics of resting splenic B cells. TI-1 antibody responses of these splenic B cells stimulated by TNP-LPS are shown in Table 4. Like the responses of their peritoneal counterparts, these cells demonstrated increased antibody secretion when IL-5 was present.

Responses to TI-2 antigens are not easily demonstrated with pure B cells due to the requirements for accessory cells. Hence, we have chosen a model system developed by others where anti-IgD coupled to high molecular weight dextran (anti-∂-dextran) is thought to mimic TI-2 antigens (*Brunswick* and *Mond*, 1988). This system allows the study of proliferation induced by this TI-2 analogue. Table 5 shows the proliferative responses of purified resting, splenic B cells stimulated by this anti-∂-dextran conjugate. Like the antibody and proliferative responses of peritoneal and splenic B cells previously shown, IL-5 was the interleukin most active at increasing the response induced by anti-∂-dextran. IL-1 showed some increase as well, but not to the same extent as that seen with IL-5.

Attempts to stimulate clonal B cell responses with the anti-∂-dextran conjugate were unsuccessful. We then tested whether DXS could provide additional signalling to anti-∂-dextran stimulated B cells. The results are presented in Table 6. Proliferation induced by different doses of anti-∂-dextran was increased by IL-5, as previously shown. However, these responses were also increased by DXS itself. Even further stimulation was seen when both DXS and IL-5 were added to anti-∂-dextran. This triple combination appeared to provide optimal signalling since responses were comparable to those seen with the anti-∂-dextran conjugate and LPS.

We attempted to quantitate resting splenic B cell precursor frequencies for responses to TI-1 and TI-2 antigens using two analogue model systems. The combination of anti-IgM+DXS was used to model TI-2 antigens and anti-IgM+LPS to model TI-1 antigens. Since these combinations are not covalently linked, their analogy to strict TI-1 and TI-2 antigens is only partial. How-

Table 6: Mitogen and Interleukin-5 enhancement of anti-∂-dextran induced proliferation of small, resting murine B cells

	Addition to culture				
Anti-∂-dextran (ng/ml)	none	IL-5	DXS	DXS + IL-5	LPS
0.0	0.5	2.0	0.9	7.7	73.4
0.01	0.5	2.9	0.8	8.3	79.7
0.1	4.0	15.3	5.5	32.3	130.1
1.0	36.0	81.8	44.5	110.2	124.5
10.0	37.0	73.0	75.4	111.1	112.9

5 x 10^4 resting, Percoll separated splenic murine B lymphocytes were cultured 2 days and pulsed with ^{3}HTdR during the terminal 6 hours of culture. Data presented are cpm x 10^{-3}. IL-5 was used at 3 U/ml, DXS at 5 μg/ml and LPS at 50 μg/ml.

ever, they do allow quantitation to be made which would otherwise not be possible without selecting antigen specific B cells and thereby perturbing the surface antigen receptors uncontrollably. Table 7 gives the summary of data from several experiments determining the frequencies of B cells proliferating in response to the two model antigen-analogue systems. Interestingly both IL-4 and IL-5 were able to increase proliferation in both systems. The IL-4 mediated increase seen with anti-IgM+DXS contrasts to that seen with the anti-∂-dextran plus IL-4.

Table 8 shows a comparison of the interleukin-mediated enhancement of proliferation induced by LPS versus that induced by anti-IgM+LPS. Whereas IL-4 increased both types of proliferative responses, IL-5 was active only when the combination of stimuli were used. Neither IL-2 nor IL-6 enhanced either of the proliferative response.

The data in the previous Tables were obtained with B cells from murine spleen and peritoneum. One is often reminded that humans differ from mice. Hence, several experiments with human B lymphocytes have been performed. Table 9 shows the responses of human peripheral blood B lymphocytes to two TI-2 analogues: anti-human IgD-dextran (anti-h∂-dex) and anti-human IgM-dextran (anti-hIgM-dex). With both TI-2 analogues, good proliferation was observed. IL-2 enhanced this proliferation whereas IL-1, IL-4, IL-5 or IL-6, did not.

The previous sections showed some differences in the responses of murine splenic and peritoneal B cells. To see if human peritoneal B cells responded differently from their peripheral blood counterparts, peritoneal fluids from patients on chronic ambulatory peritoneal dialysis (CAPD) were collected. While cells from these fluids come from patients, they represent one of the few sources of such cells and so, although not derived from normal volunteers, were used as a first estimate of human peritoneal lymphocyte responsiveness.

Preliminary experiments revealed no responsiveness to B cell stimulating agents (data not shown). This is probably explained by the paucity of B cells in these peritoneal dialysate preparations, as revealed in Table 10. This table shows the prevalence of cells exhibiting B and T lymphocyte markers in 5 CAPD patients. As can be seen, only one patient showed significant B cell levels and this patient, although showing no overt signs of inflammation, was clearly different from the other patients in the population. Nevertheless, T cell reactivity, especially to the bacterially

Table 7: Interleukins increase clonal B cell growth initiation by anti-IgM plus dextran sulphate and by anti-IgM plus LPS

Stimuli	Addition	Average frequency (%) of B cells yielding clones	s.e.
anti-IgM + DXS	none	14.5	5.9
	IL-1	24.4	7.0
	IL-4	29.4	7.9
	IL-5	21.1	6.8
anti-IgM + LPS	none	32.1	6.8
	IL-4	70.6	10.7
	IL-5	51.0	9.0

Resting murine splenic B cells were cultured with the indicated stimuli and Poisson analyses were performed to determine the fraction of input B cells stimulated to proliferate. These are presented as percentages of the input B cells which responded. Shown also are the associated standard errors. Four experiments were performed for anti-IgM + DXS and ten experiments for anti-IgM + LPS. LPS was used at 50 μg/ml, DXS at 5 μg/ml and anti-IgM was 1 μg/ml of the b-7-6 monoclonal rat anti-mouse IgM antibody.

derived superantigen staphylococcal enterotoxin B (SEB), could be reproducibly detected in peritoneal cell preparations from these patients. This is shown in Table 11 where proliferation responses induced by a T cell mitogen and SEB can be observed.

DISCUSSION

The experiments presented in the previous sections provide clear evidence that T cell products can regulate B cell responses to thymus independent activators and antigens. Local immune responses to TI-1 antigens by murine peritoneal B cells were increased by IL-5. Similarly, murine splenic B cell responses to TNP-LPS were also increased by IL-5. These data suggest a role for IL-5 in murine responses to TI-1 antigens. Interestingly, the data in Table 8 suggest that resting B cells must perceive surface antigen receptor cross-linking as well as a second polyclonally activating signal to induce IL-5 responsiveness. The data in Tables 5, 6 and 7, using anti-∂-dextran and anti-μ + DXS as models of TI-2 antigens, and with anti-μ + LPS as a TI-1 analogue model support this interpretation. We (*Wetzel*, 1991a, 1991b) and others (*Pike* et. al., 1987) have also found a role for IL-5 and IL-1 in supporting murine B cell responses driven by TI-2 antigens.

The data in Table 6 suggest that there may be several forms of TI-2 antigens. Addition of DXS to anti-∂-dextran increased the responses observed although not obviously changing the quality of these responses, i.e. IL-5 increases responses to both agents. Furthermore, DXS alters the response to anti-IgM by allowing clonal B cell proliferation in the presence of the combination of anti-IgM+DXS, as shown in Table 7. Again, IL-5 is active in this system but IL-4 is now also active. DXS by itself is a weak activator, as shown in Table 1, and hence, although apparently polyclonally activating, may best be classified as TI-2 type in its action. These properties of DXS are inter-

Table 8: Determination of the proportion of small, resting B cells proliferating in response to thymus regulated antigen type 1 analogues plus interleukins

Added interleukin	Primary stimuli	
	PLS	LPS + anti-IgM
none	2.7 ± 2.1	20.1 ± 5.4
IL-2	3.2 ± 2.2	11.6 ± 4.0
IL-4	29.0 ± 6.9*	60.2 ± 11.5*
IL-5	5.4 ± 2.7	37.5 ± 8.2*
IL-6	1.5 ± 1.7	21.9 ± 5.7

Percoll separated small, resting, murine splenic B cells were cultured in Terasaki plates at four different cell inputs (from 1 to about 8 cells/well, with no filter cells) with 60 wells/input dose for each determination. After 4 days, wells were examined for cell growth and Poisson analysis was used to calculate the frequency of input B cells stimulated to proliferate. These frequencies are presented as percentages of the input B cells (i.e. a frequency of 0.1 = 10%) and are given with the associated 95% confidence intervals.
*Asterisks mark those frequencies which are statistically different from the appropriate controls at less than the 5% level.
LPS was used at 50 μg/ml and the b-7-6 monoclonal anti-IgM used at 10 μg/ml. Interleukins were used at 10 U/ml except for IL-6 which was used at 1000 U/ml.

esting but it remains difficult to interpret them before a more complete understanding of how DXS interacts with B cells is available. In any case, the data are suggestive that more than one form of TI-2 antigen may exist. Furthermore, the data suggest that different interleukins can be important in responses to different forms of TI antigens.

The responses by human B cells revealed different interleukin receptivities from those seen with mouse B cells. Proliferation by human peripheral blood B lymphocytes induced with anti-hδ-dextran and anti-hμ-dextran was increased by IL-2 but not the other interleukins tested, as seen in Table 9. As described above, mouse B cells show different reactivities. The reason for this difference is not immediately obvious. Nevertheless, B cells from both species reveal interleukin receptiveness when stimulated by TI-2 analogues and therefore provide evidence that T cells can regulate and may even be required for TI-2 responses in some situations.
The difference between species in interleukin responsiveness may parallel the difference seen between B cells from different tissues in the mouse. Murine peritoneal B cells respond to IL-5 by itself, as seen in Tables 1 and 2 and elsewhere (*Wetzel*, 1989, 1990, 1991c), although these responses are small. Induction of resting splenic B cell IL-5 responsiveness requires surface receptor engagement and perception of a polyclonally activating stimulus. Hence, it may be possible to reveal human B cell IL-5 responsiveness with the appropriate stimulus. Our initial attempts to reveal human B cell IL-5 responsiveness using peritoneal cell populations were unsuccessful due to the lack of B cells in these populations. Nevertheless, human B cell IL-5 responses have been observed by others using some appropriate bacterial activators (*Mond* and *Harriman*, personal communications).

The finding of T cell responsiveness to SEB in lymphocyte preparations from the peritonea of CAPD patients, seen in Table 11, shows at least some functional and responsive anti-bacterial immune mechanisms in this human or-

Table 9: Stimulation of human peripheral blood B lymphocytes by the thymus independent type 2 antigen analogues anti-IgD-dextran and anti-IgM-dextran

		cpm/culture x 10^{-3}					
		Addition					
Exp.	Stimulus	none	IL-1	IL-2	IL-4	IL-5	IL-6
1	medium	0.5	0.4	1.2	0.3	ND	ND
	anti-∂-dextran	36.6	51.1	64.9	28.1	ND	ND
2	medium	5.9	7.3	12.6	ND	6.6	ND
	anti-∂-dextran	65.8	59.4	90.1	ND	55.2	ND
3	medium	4.3	4.6	12.7	ND	ND	1.8
	anti-∂-dextran	14.1	10.8	37.0	ND	ND	7.1

5 x 10^4 human peripheral blood B lymphocytes were cultured 2 days (experiment 1) or 3 days (experiments 2 and 3) with 0.1 μg/ml of the indicated anti-immunoglobulin-dextran conjugate and pulsed for the terminal 16 hours of culture with tritiated thymidine. IL-1 was used at 100 U/ml, IL-2 at 30 U/ml, IL-4 at 20 U/ml, rIL-5 at 200 pg/ml and IL-6 at 1000 U/ml.

gan. These results, coupled with those from the previous tables, show the importance of bacterial immunity. Both B cells and T cells have developed mechanisms to deal with this type of insult. It appears that each of these lymphocyte populations can respond independently as well as communicate with each other. Thus, T cells are capable of regulating B cell responses to TI antigens. The specific mediators developed for these regulatory interactions can vary according to the type of stimulation encountered by the B cell and by the tissue or species of B cell origin. It is probable that the complexity of these regulatory networks and responding populations is only beginning to be appreciated.

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Table 10: Phenotypic analysis of lymphoid cells from peritoneal fluids of patients on continuous ambulatory peritoneal dialysis

	% of cells analysed expressing the indicated marker					
	Patient number					Monocyte depleted
Marker	1	2	3	4	5	blood
CD3	38.7	51.2	34.5	9.7	9.8	59.1
CD4	23.7	34.8	12.4	ND	ND	21.6
CD19	2.0	0.1	0.1	0.0	0.7	20.1
CD20	0.6	1.3	ND	ND	ND	7.9
LeuM3	35.6	12.4	9.1	12.1	5.2	0.3

Cells from peritoneal fluids were recovered by centrifugation and then stained with the appropriated fluorescently labelled monoclonal antibodies. Cells were then analysed on a FACSCAN and the numbers shown are the percentages of either small cells or of total cells positive for binding the indicated fluorescent antibodies. Anti-CD3 and anti-CD4 monoclonals detect all and helper T cells, respectively. Anti-CD19 and anti-CD20 monoclonal antibodies detect human B cells. Anti-LeuM3 detects human monocytes and macrophages.

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Table 11: Proliferative responses of cells recovered from peritoneal fluids from patients on continuous ambulatory peritoneal dialysis

		cpm x 10^{-3}				
		Interleukin added to culture				
Exp.	Stimulus	None	IL-1	IL-2	IL-5	IL-6
1	medium	0.7	1.5	2.7	1.7	1.5
	SAC	1.5	1.7	1.9	1.9	0.9
	SEB	4.1	4.5	6.7	4.5	4.8
2	medium	0.3		0.8		
	SEB	3.6		6.2		
	PHA-P	1.2		1.8		
3	medium	0.4		1.3		
	SEB	4.0		7.1		
	PHA-P	1.5		2.9		

All experiments were performed with cultures of L-Leucil Methyl Ester treated with peritoneal dialysis fluid cells. Experiment 1 used 1000 cells per well and experiments 2 and 3 used 500 cells per well. Experiment 1 was assayed on day 3 of culture whereas experiments 2 and 3 were assayed at day 5. Cultures were pulsed with 1 μCi of ^{3}HTdR for 16 hours prior to harvest and incorporated radioactivity was measured by liquid scintillation counting. *Staphylococcus aureus* protein A (SAC) was used at 10 μg/ml. Staphylococcal enterotoxin B (SEB) was used at 10 ng/ml and Phytohaemagglutinin-P (PHA-P) was used at 2 μg/ml. Interleukins 1, 2, 5 and 6 were used at 100 U/ml, 30 U/ml, 200 pg/ml, and 1000 U/ml, respectively.

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