AN ABSTRACT OF THE THESIS OF

Sharon Levy for the degree of Master of Science in Toxicology presented on April 6, 1988. Title: The Effect of 3,3',4,4',5,5'-Hexachlorobiphenyl on Production of Interleukins 1 and 2 and Lymphocyte Proliferation in an Allogeneic Response.

Abstract approved: Redacted for privacy

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In vivo generation of cytotoxic T cell activity against alloantigen is suppressed by the aromatic hydrocarbon (Ah) receptor binding 3,3',4,4',5,5'-hexachlorobiphenyl isomer (HxCB). Previous studies in this laboratory suggest that HxCB may alter early event(s) in the activation of cytotoxic T lymphocytes (CTL). Production of and response to interleukins 1 and 2 are crucial early events in the generation of effector CTL. In the present study, models were developed to evaluate interleukin 1 (IL 1) production, interleukin 2 (IL 2) production, and the proliferative response to IL 2 in the HxCB treated and control C57B1/6 mice. The model system used throughout was the rejection of the allogeneic P815 mastocytoma. In the case of IL 1 production, the ability of peritoneal exudate cells

(PEC) to respond to *in vitro* stimulation with bacterial lipopolysaccharide (LPS) was also examined. Likewise, IL 2 production in response to Conconavalin A (Con A) was tested. Results suggested there was no significant effect of HxCB on production of either interleukin. The ability of lymphocytes from allosensitized animals to proliferate in response to IL 2 also appeared to be unaffected by HxCB exposure in the experimental model presented here. Thus, while other work in this laboratory suggests that HxCB prevents the normal activation and/or proliferation events that occur in the response to P815 challenge, the production of IL 1 and IL 2 and proliferative response to IL 2 do not appear to be targeted by HxCB.

The Effect of 3,3',4,4',5,5'-Hexachlorobiphenyl on Production of Interleukins 1 and 2 and Lymphocyte Proliferation in an Allogeneic Response

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed April 6, 1988

Commencement June 1989

APPROVED:

Redacted for privacy

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Redacted for privacy

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Date thesis is presented April 6, 1988

Typed by Deborah Safley for Sharon Levy

ACKNOWLEDGEMENTS

Thanks to Dr. Nancy Kerkvliet, Linda Steppan and Julie Brauner for sharing their knowledge, patience and humor during the time I worked in their lab. Technical help and advice were generously given by Dr. Robert House and Karen Richardson. Special thanks to Dr. Paul Conlon and Sue Tyler of Immunex Corporation for testing some of my samples.

Neither I nor my thesis would exist in any coherent form without Paula, Sol, Florence, and David, people of warmth, wit and perspective. Thank you Lindsay for friendship and strategic advice, and to Jim: here's looking at you, kid.

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INTRODUCTION

Polychlorinated biphenyls (PCBs) are widespread environmental pollutants. Some of the same physical/chemical properties of PCBs which make them useful in industry contribute to making them a problem in the environment. These properties include resistance to temperature changes, acid, alkali, oxidation and hydrolysis, high electrical resistance and low solubility in water. PCBs are lipophilic and therefore are bioconcentrated. They have been detected in air, water, and animal tissues around the world (Verschueren, 1983). PCBs have been used in a number of industrial applications, including electrical capacitors and transformers, lubricating and cutting oils, adhesives, plastics, inks, paints and sealants. Commercial PCB mixtures contain a variety of PCB isomers as well as polychlorinated dibenzofuran contaminants.

Like certain other halogenated aromatic hydrocarbons (HAH), PCBs have been shown to be

immunosuppressive. The mechanism of immunosuppression remains unclear. Both humoral and cell-mediated immunity can be suppressed by PCBs. PCBs appear to concentrate in lymphoid tissue, sometimes to an even greater extent than in adipose tissue (Bleavins & Aulerich, 1983).

Previous results have demonstrated that PCB suppresses the in vivo generation of CTL if the exposure takes place early in the immune response (Kerkvliet & Baecher-Steppan, in press). If PCB exposure is delayed until effector CTL are present, no suppression is seen. These experiments suggest that PCB affects some early step(s) in the complex series of events leading to the generation of effector CTL.

The generation of and response to interleukins 1 and 2 are crucial early events in the cell mediated immune response. In this project the effect of in vivo exposure to PCB on the ability to produce and respond to interleukins 1 and 2 was investigated. The model system used was the CTL response to an allogeneic ascites tumor, the P815 mastocytoma.

T Cell Activation and the Role of Interleukins 1 and 2

IL 1 was originally described as a factor produced by macrophages which was mitogenic for T lymphocytes

(Gery et al., 1972; Gery & Waksman, 1972). It is now understood to have a wide variety of activities on different cell types throughout the body and to be a crucial factor in inflammation and induction of fever. Most relevant to this study is the role of IL 1 in the activation of T cells.

The discovery of IL 2 arose from the experimental observation of T cell-T cell interactions in graft vs. host responses and in primary mixed lymphocyte cultures (MLC) (Wagner et al., 1980). The addition of syngeneic thymocytes to peripheral T cells in MLC resulted in greatly increased CTL responses. This effect was found to be mediated by a soluble factor produced by T helper cells which amplified the response of effector CTL.

Since the early 1970's a great deal of work has been done to elucidate the molecular characteristics, production signals and function of interleukins 1 and 2. While the understanding of T cell activation continues to evolve, the importance of both these factors in the process of T cell activation is well established.

Both interleukins have been isolated. IL 1 is a polypeptide. The secreted form is about 15 kD in all species tested. The 15 kD peptide is the result of post-translational processing of a larger precursor form of 35 kD. IL 1 may be a glycoprotein, although there is

no direct evidence that carbohydrates are present or necessary for IL 1 activity (Durum, Schmidt, & Oppenheim, 1985). IL 2 is a glycoprotein of about 15 kD. The formation of agregates or the degree of glycosylation may cause variations in molecular weight without significantly affecting its biological activity (Forni et al., 1986).

Both interleukins are antigen-nonspecific mediators but are essential activating signals in T cell dependent, antigen-specific responses as well as the response to T cell mitogens such as Con A and phytohemagglutinin (PHA) (Forni et al., 1986; Nabholz & MacDonald, 1983; Mizel, 1982).

Macrophages are the major IL 1 producing cells of the immune system. At least two separate macrophage signals are needed for antigen-specific T cell activation. One is antigen presented on the macrophage surface in the context of I region associated (Ia) macromolecules, and the other is IL 1 (Mizel, 1982; Durum et al., 1985). These two signals induce both the expression of receptors for IL 2 and the production of IL 2 in T cells. IL 1 has also been shown to augment PHA-induced production of T cell mRNA for IL 2, interleukin 3 and the cell surface macromolecule Ly-1, which is associated with T cell activation (Hagiwara et

al., 1987). Macrophage production of IL 1 can be induced by a variety of stimuli including alloantigen, bacteria, viruses, immune complexes, LPS, phorbol myrstic acetate, Ia-restricted cell to cell contact with activated T cells, or by soluble factors produced by activated T cells (Weaver & Unanue, 1986; Durum, Schmidt, & Oppenheim, 1985).

A second set of signals, consisting of appropriately presented antigen and IL 2, induces T helper cell proliferation and maturation of CTL precursors (CTLp) to effector CTL. IL 2 is necessary for the passage of activated cells (in G1 phase, induced by interaction of antigen or lectin with the T cell antigen receptor) into S phase (Fletcher & Goldstein, 1987; Nabholz, & MacDonald, 1983).

The IL 2 receptor (IL2R), a 55 kD glycoprotein, is expressed early in the response to antigen, before IL 2 is available (Fletcher & Goldstein, 1987). There are two affinity classes of IL2R. The number of high affinity receptors occupied closely correlates with the magnitude of the T cell response. Binding of IL 2 to high affinity IL2R induces the expression of additional IL2R at the cell surface. The extent of IL 2 secretion and IL2R expression determine the expansion and

subsequent termination of the T cell response (Waldmann, 1986).

Although this view of the role of IL 2 was initially developed on the basis of *in vitro* experiments, recent *in vivo* studies substantiate it. Cells expressing IL2R are present in normal primary and secondary lymphoid organs (Forni et al., 1986). Monoclonal antibodies to IL 2 or IL2R inhibit allograft responses both *in vitro* and *in vivo* (Forni et al., 1986).

IL 2 enhances the production of gamma interferon (IFN), B cell growth factor(s), and granulocyte-macrophage colony stimulating factor, but does not appear to regulate its own production (Waldmann, 1986). *In vivo* IL 2 activity is regulated by inhibitors in serum and the shedding of IL2R. These, in combination with the short halflife of IL 2 *in vivo*, prevent locally induced IL 2 from exercising widespread systemic effects (Forni et al., 1986). In contrast, IL 1 can act either as a local mediator or as a circulating hormone (Durum, Schmidt, & Oppenheim, 1985).

The production of IL 1 is regulated in part by activated T cells. T cells responding to soluble protein antigens or to alloantigen have been shown to produce a mediator which increases IL 1 production by

macrophages (Weaver & Unanue, 1986; Dinarello & Kent, 1985). This factor is still undefined, but it does not appear to be IL 2, IFN, B cell stimulating factor(s) or colony stimulating factor. IFN produced by activated T cells upregulates macrophage expression of Ia glycoproteins, which enhances the macrophage's ability to present antigen. Thus there appears to be a positive feedback loop in T cell activation.

IL 1 also contributes to its own regulation by a direct effect on macrophages. Macrophages respond to IL 1 by releasing prostaglandin E2 (PGE2) which inhibits both IL 1 production and the expression of Ia glycoproteins (Durum, Schmidt, & Oppenheim, 1985).

T Cell Subpopulations

Ideas concerning the subpopulations of T cells responsible for different T cell functions (T helper cells vs. effector CTL) have changed over time. The dogma stating that T helper cells and effector CTL belong to phenotypically distinct subpopulations (Henney, Kern, & Gillis, 1982) has proven to be an oversimplification. L3T4+ and Ly 2+ T cells can each function as either helper or effector cells (Hao et al., 1987). Ly 2+ cells, traditionally thought of as effector CTL requiring help from the L3T4+ subset, have been shown to be able to produce IL 2 and to proliferate

in response to Con A in the absence of L3T4+ cells (Kern, & Lachmann, 1987). Rather it is the class of MHC antigen recognized by the cell which is determined by possession of L3T4 or Ly 2 surface markers. L3T4+ cells recognize Class II MHC antigens while Ly 2+ cells recognize Class I.

Similarly, the requirement for L3T4+ cells in _in vivo_ allograft rejection is not absolute, but depends on the type of tissue transplanted and the MHC differences between donor and recipient (Hao et al., 1987). Primed Ly 2+ cells are capable of rejecting allografts with a Class I MHC difference in the absence of L3T4+ cells. Rejection of allografts expressing only a Class II or minor histocompatibility difference is, however, dependent on L3T4+ cells.

The stimuli needed to induce IL 2 production in Ly 2+ cells are different from those needed to induce cytolytic function. Contact with macrophages is necessary to induce helper function in both L3T4+ and Ly 2+ cells stimulated by Con A, while macrophages are not required for proliferation or CTL generation by Ly 2+ cells when exogenous lymphokines are provided (Kern & Lachmann, 1987).

Inhibitors of IL 1/IL 2 Production and Action

Considering the critical role of interleukins 1 and 2 in T cell activation, it is not surprising that deficiencies of one or both lymphokines are involved in immunosuppression caused by certain chemicals or diseases affecting the immune system. In Hodgkin's disease, acute graft vs. host disease, systemic lupus erythematosus, and AIDS, there is a deficiency in IL 2 production (Fletcher & Goldstein, 1987). Because IL 2 contributes to the regulation of IL2R expression, a lack of IL 2 can cause suboptimal IL2R expression. This leads to reduced cellular proliferation and effector function in T cell populations.

A number of drugs and chemicals have been shown to affect the production or function of interleukins 1 and 2. Cyclosporin, which is used as an immunosuppressant in patients receiving organ transplants, blocks the production of both lymphokines but does not affect IL2R expression (Fletcher & Goldstein, 1987; Durum, Schmidt, & Oppenehim, 1985). The polycyclic aromatic hydrocarbons (PAH), widespread environmental contaminants which are immunosuppressive, may exert their effects by inhibiting lymphokine production. Dimethylbenzanthracene suppresses IL 2 production by mitogen stimulated splenocytes (House, Lauer, Murray, &

Dean, 1987). There is some evidence that benzo(a)pyrene, another PAH, may affect the production of IL 1 by macrophages (Lyte & Bick, 1986).

Corticosteroids are endogenous immunosuppressants which inhibit the production of both IL 1 and IL 2, but do not affect IL2R expression (Fletcher & Goldstein, 1987). There appears to be a direct effect of corticosteroids on both macrophages and T helper cells (Palacios, 1982) so that the IL 2 deficit induced by corticosteroids is not due entirely to a decrease in available IL 1.

PCB exposure elevates serum corticosterone levels, which suggests that PCB-induced inhibition of the CTL response might be an indirect effect due to increased corticosterone. This possibility is currently under investigation in this laboratory (Kerkvliet, Smith & Steppan, 1988).

HAH Toxicity and the Ah Receptor

PCBs belong to a general class of chemicals called HAH. In addition to their similar chemical structures, they also produce a characteristic pattern of toxic effects and are believed to act by a common mechanism (Poland & Knutson, 1982). 2,3,7,8-tetrachlorodibenzo-p-

dioxin (TCDD) is the prototype chemical of the group, and the most potent toxicant.

Different animal species have widely varying sensitivities to HAH, and the symptoms of toxicity also vary among species. However, biologically active HAH congeners will generally cause the same pattern of toxic responses in a given species (Poland & Knutson, 1982). The order of species sensitivity to HAH toxicity will also be similar for all active congeners.

Typical effects produced by HAH exposure include cancer promotion, hepatotoxicity, induction of microsomal enzyme activity, chloracne or other epidermal changes, gastric and urinary tract lesions, and lymphoid involution (Poland & Knutson, 1982). TCDD and toxic PCB isomers cause thymic atrophy in all species tested, as well as a loss of lymphoid tissue in the spleen and lymph nodes in most species (Poland & Knutson, 1982; Kerkvliet, 1984). It was this effect on lymphoid tissue which originally led to the investigation of the immunotoxic potential of HAH. TCDD and congeners have been shown to suppress both humoral and cell mediated immunity (Kerkvliet, 1984).

These toxic effects are part of a set of coordinately expressed responses induced when HAH isomers bind to a cytosolic protein receptor termed the

Ah receptor. Included in this set of responses is induction of microsomal mixed function oxidase (MFO) activities. These enzymes act to metabolize foreign lipophilic chemicals into more polar and therefore more readily excreted compounds. Different HAH isomers induce different isozymes with varying substrate specificities. The HAH have been divided into three classes of MFO inducers, based on the types of MFO activity induced by 3-methylcholanthrene (MC) and phenobarbitol (PB). The extent of aryl hydrocarbon hydroxylase (AHH) activity is often used as a measure of MC type induction, whereas aminopyrine-N-demethylase (APND) activity is used to indicate PB type induction (Silkworth, Antrim & Kaminsky, 1984). A chemical which elicits isozymes induced by both MC and PB is called a mixed type inducer. PB type inducers do not act via the Ah receptor. TCDD, as well as several toxic PCB isomers, are MC type inducers and bind the Ah receptor (Poland & Knutson, 1982).

For TCDD and related HAH, there is a strong correlation between the toxicant's affinity for the Ah receptor and its potency for both toxicity and the induction of AHH (Poland & Knutson, 1982; Silkworth & Vecchi, 1985). The Ah receptor was first studied in mice, following the discovery that a certain inbred

mouse strain, DBA/2, failed to respond to MC with increased MFO activity. These mice are also highly resistant to HAH induced toxicity. Randomly bred and some inbred mice do respond to MC with MFO induction and are susceptible to HAH toxicity. The trait of responsiveness is inherited as a simple autosomal dominant (Poland & Knutson, 1982). The genetic locus coding for this trait is designated the Ah locus. DBA/2 is the prototype nonresponder mouse strain, with genotype Ah^{d}/Ah^{d}. C57Bl/6 is the prototype responder strain, with genotype Ah^{b}/Ah^{b}. Nonresponder strains express an Ah receptor of lower affinity for TCDD and similar ligands. TCDD can produce toxic effects in DBA/2 mice, but at doses 10-100 times higher than are required to produce similar effects in C57Bl/6 mice. The discovery of an inbred strain possessing such a low affinity Ah receptor made it possible to study the role of this receptor in the induction of both MFO activities and toxicity.

The Ah receptor has been found in the tissues of many species and strains of animals (Vos, 1977; Kerkvliet, 1984). It has been identified in the cytosol of liver, lung, thymus, kidney, brain, and skeletal muscle, with highest concentrations in lung and thymus (Silkworth & Vecchi, 1985).

When HAH bind to the receptor, the receptor-ligand complex is translocated to the nucleus. There the complex interacts with DNA, causing activation of genes controlling the expression of MFO activities and other gene products (Poland et al., 1979).

Immunosuppression, like other types of HAH induced toxicity, correlates with the isomer's affinity for the Ah receptor and is strain dependent in mice. Ah-interacting HAH isomers which are immunosuppressive in C57Bl/6 mice require much higher doses to produce a comparable effect in DBA/2 mice (Silkworth, Antrim & Kaminsky, 1984; Silkworth & Vecchi, 1985). These findings support the idea that activation of the Ah gene complex is a requirement for immunotoxicity. The role of the Ah receptor in immunotoxicity remains unclear, however. Coordinately expressed induction of MFO activities does not appear to be a direct cause of immunosuppression or lymphoid involution (Poland & Knutson, 1982; Kerkvliet, 1984). Several authors have suggested that protein products induced by activation of the Ah gene complex could affect cellular differentiation (Poland & Knutson, 1982; Greenlee et al., 1984). This is a possible mechanism of immune suppression via the Ah receptor, since cellular

differentiation and proliferation are crucial to immune responses.

Structure-Activity Relationship for PCBs

For PCBs as for other HAH, toxicity depends on the positions and number of halogen substitutions. The halogen substitution pattern determines the conformation of the molecule, and therefore its ability to bind the Ah receptor (Silkworth & Vecchi, 1985). Toxic PCB isomers have four lateral chlorines arranged in an approximately 3 x 10 Angstrom box. Net polarizability of the molecule underlies the apparent requirement for symmetry within the molecule (McKinney & Singh, 1981). Sufficient polarization for binding of the Ah receptor requires four lateral chlorines. Coplanarity of the phenyl rings is necessary to achieve juxtaposition of the four lateral chlorines, and planar isomers generally exhibit higher affinity for the receptor than do nonplanar isomers (McKinney & Singh, 1981; Silkworth & Vecchi, 1985).

Commercial PCB mixtures contain a number of PCB isomers of varying biological activities. PCB mixtures such as Aroclor 1254 have been shown to cause immune dysfunction in several species known to express the Ah receptor (Silkworth & Vecchi, 1985). Isomers such as

3,3',4,4'-tetrachlorobiphenyl, 3,3',4,4',5,5'-hexachlorobiphenyl and 2,3,3',4,4',5-hexachlorobiphenyl, which meet the above stated requirements for binding the Ah receptor, impair both humoral and cell mediated immunity (Silkworth, Antrim & Kaminsky, 1984; Clark, Sweeney, Safe, Hancock, Kilburn & Gauldie, 1983). Asymmetrical isomers like 2,2',4,4'-tetrachlorobiphenyl and 2,2',4,4',6,6'-hexachlorobiphenyl do not bind the Ah receptor and are not immunosuppressive.

PCB Effects on Immunity

Generalized signs of immunotoxicity induced by PCB exposure include lymphopenia, lymphoid tissue hypoplasia, bone marrow hypocellularity, and involution of primary and secondary lymphoid organs (Kerkvliet, 1984). Humans poisoned with PCB-contaminated oil showed increased susceptibility to respiratory infections, in addition to chloracne and other symptoms (Dean, Murray & Ward, 1986.). Specific effects of PCBs on the humoral and cell mediated arms of the immune response are discussed below. Alteration of specific immune functions can occur at levels of PCB exposure which do not cause overt toxic signs such as thymic atrophy or lymphoid cell depletion.

PCB Effects on Humoral Immunity

PCBs have been shown to cause reduction in bursa weight in chickens and suppression of the antibody response to T dependent antigens in mice, guinea pigs, and rhesus monkeys (Kerkvliet, 1984; Bleavins & Aulerich, 1983). The antibody response to T independent antigens is also reduced by PCB exposure (Silkworth & Antrim, 1985). PCB treated mice show increased susceptibility to endotoxin and to Salmonella infection.

Humans suffering from PCB poisoning had significantly decreased levels of IgM and IgA in their serum (Lu & Wu, 1985). Peripheral blood lymphocytes from these subjects showed an enhanced proliferative response to the B and T cell stimulating pokeweed mitogen (PWM). However, peripheral blood lymphocytes from farmers exposed to high levels of polybrominated biphenyls, a related HAH, showed a decreased response to PWM (Bekesi et al., 1979).

PCB Effects on Cell Mediated Immunity

Delayed type hypersensitivity reactions in rabbits, guinea pigs, rodents and humans are suppressed by PCBs (Kerkvliet, 1984; Chang, Hsieh, Lee, Tang & Tung, 1981).

Challenge of PCB exposed animals with infectious agents has shown decreased host resistance in ducks to

duck hepatitis virus and in mice to herpes simplex virus, ectromelia virus, and Plasmodium berghei (Dean, Murray & Ward, 1986).

Human peripheral blood lymphocytes exposed to PCBs in vitro show a reduced response to PHA, a T cell mitogen (Bleavins & Aulerich, 1983). However, peripheral blood lymphocytes of PCB poisoned patients showed increased spontaneous proliferation as well as augmented responses to the T cell mitogens PHA and Con A (Wu, Hsieh & Lu, 1984). Peripheral blood lymphocytes of rats exposed to Aroclor 1254 also had an increased proliferative response to PHA (Bonnyns & Bastomsky, 1975). In another study, splenocytes of rats given Aroclor 1254 produced higher levels of IL 2 in response to Con A than did controls, while in vitro exposure to Aroclor decreased IL 2 production (Exon, Talcott & Koller, 1985). The mechanism augmentation of in vitro lymphocyte proliferative responses following in vivo exposure to PCBs is still unexplained.

Changes in the subpopulations of T lymphocytes have been observed in PCB poisoned patients. These include a decrease in the total percentage of T cells in peripheral blood as well as a decrease in the T helper cell/T cytotoxic cell ratio (Wu, Hsieh & Lu, 1984; Chang, Hsieh, Lee, Tang & Tung, 1981).

PCBs inhibit the in vivo generation of CTL against alloantigen (Clark et al., 1983; Kerkvliet & Baecher-Steppan, in press). The response of C57Bl/6 mice to P815, an allogeneic mastocytoma, is the model system used in this project.

Mechanism of PCB Immunotoxicity

The mechanism of HAH induced immunotoxicity remains unclear. Recent work in this area has generated conflicting theories.

Some researchers have suggested that the effects of HAH on lymphocytes are indirect and are mediated by a direct effect on the epithelium of the thymus (Clark, Gauldie & Sweeney, 1984; Greenlee et al., 1984). TCDD may act on thymic epithelial cells with the result that thymus-dependent maturation of T lymphocyte precursors is altered (Greenlee, Dold, Irons & Osborne, 1985). Clark et al., attribute immunosuppression caused by TCDD exposure to the induction of T suppressor cells in the thymus (Clark, Sweeney, Safe, Hancock, Kilburn & Gauldie, 1983). A study of TCDD effects on murine bone marrow chimeras lends support to the idea of an immunosuppressive effect mediated by the thymic epithelium. Susceptibility to immune suppression by TCDD was determined by the Ah genotype of the

nonlymphoid cells of the host rather than by that of the grafted lymphoid cells (Nagarkatti, Sweeney, Gauldie & Clark, 1984).

This view, however, does not account for the HAH induced suppression of the antibody response to T independent antigens in vivo, or the suppression of B cell differentiation in vitro (Tucker et al., 1986). Furthermore, it has been demonstrated that Ah interacting PCB isomers can produce immune suppression without thymic atrophy (Silkworth & Vecchi, 1985), and that adult-thymectomized mice are suppressed to the same extent as controls with intact thymus (Kerkvliet & Baecher-Steppan, in press). Therefore, an effect of HAH on the thymic epithelium cannot fully explain the mechanism of immunotoxicity. There may be several different ways in which Ah receptor ligands can exert their immunotoxic effects.

Studies of PCB immunosuppression in this laboratory have focused on HxCB, a single purified isomer of relatively high toxicity and affinity for the Ah receptor. The literature on PCB immunotoxicity has often contained conflicting results. The use of commercial PCB mixtures containing various isomers in differing amounts has contributed to this problem. In order to study the mechanism of PCB immunosuppression,

it is necessary to use a purified isomer of known characteristics.

Previous studies have shown that HxCB produces a dose dependent suppression of CTL activity generated in vivo against P815, and that this suppression is not due to a shift in the kinetics of the cytotoxic response (Kerkvliet & Baecher-Steppan, in press). In P815 sensitized male C57bl/6 mice, 10 mg/kg HxCB also significantly suppresses and the splenic enlargement and increase in spleen cellularity which normally occur in response to P815 challenge.

HxCB exposures taking place from 6 weeks before P815 injection up to 3 days after injection significantly suppress cytotoxicity. If HxCB exposure occurs more than 3 days after P815 injection, CTL activity is not significantly suppressed. These timing studies suggest that HxCB alters some early event in the activation of CTL. The mechanism of immunotoxicity may perhaps be better understood by determining at what step(s) HxCB alters the early events of the immune response.

Using the model already developed in this laboratory of HxCB-induced suppression of the CTL response, this study investigated the possible effects

of HxCB on the production of and/or response to interleukins 1 and 2.

MATERIALS AND METHODS

Mice

Five-week old male C57bl/6 mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were maintained in groups of 5 or 6 in plastic cages with dust-free alder shavings and given Wayne Rodent Chow and tap water ad libitum.

PCB Exposure

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(3,4,5) HxCB was purchased from Ultrascientific, Hope, Rhode Island. The chemical was dissolved in acetone and diluted in peanut oil. The acetone was then evaporated from the solution using nitrogen gas. A vehicle-only solution was prepared in a similar manner. Mice were dosed orally by stomach tube once with 10 mg/kg of HxCB on the day before alloimmunization.

Alloimmunization

The P815 mastocytoma was maintained in an ascites form by weekly passage in DBA/2 mice, the strain of origin. B6 mice were alloimmunized by intraperitoneal injection of 1×10^7 viable P815 cells.

Incubation of Cell Cultures

All cell cultures were incubated in a Narco Model 6300 incubator at 37 ^{o}C and an atmosphere of 5% CO_2.

IL 1 Production Models

Ex vivo model

Alloimmunized, HxCB-exposed and control mice were sacrificed by CO_2 asphyxiation at 6, 12, 24, 54, 72 and 96 hours after injection of P815. Nonimmune control groups, which received only vehicle in place of both P815 and HxCB, were run concurrently and sacrificed at 12, 54 and 96 hours. There were 4-5 mice per treatment group. Peritoneal exudates were collected by lavage, using 5 ml per mouse of Iscove's Modified Dulbecco's Medium with 10% FBS, heparin 5 U/ml, and 20 ug/ml gentamicin (10% IMDM). Cell concentrations were determined by use of a Coulter cell counter. Peritoneal exudates were incubated 24 hr in Linbro 24 well flat bottom tissue culture plates (#76-033-05), at a volume of 1.5 ml/well. After 24 hr, supernatants were harvested and centrifuged at 900 x g for 15 minutes, then stored at 4 ^{o}C.

Cytocentrifuge slides of the PEC were taken prior to incubation of the samples, using a Shandon Cytospin centrifuge. Slides were stained consecutively with

Gugol and Giemsa stains for the microscopic differentiation of cells into lymphocytes, macrophages, tumor cells and granulocytes.

In order to determine whether IL 1 detected might have been produced by the P815 cells themselves, supernatants of P815 cell cultures were collected and assayed for IL 1. P815 cells taken from a DBA host by lavage were incubated at 10^6 cells/ml and 2×10^5 cells/ml for 24 and 48 hrs before harvest of supernatants.

In vitro model

HxCB-exposed and control B6 mice, 7 per treatment group, were injected i.p. with 1.5 ml/mouse of aged thioglycollate medium on the day following intubation. On day 3 after thioglycollate injection, PEC were harvested by lavage using 8 ml/mouse of RPMI 1640 with 10% FBS, 10 mM Hepes buffer, and 20 ug/ml gentamicin (10% RPMI). Cells were washed once and cell concentrations determined using the Coulter cell counter. Cells were suspended at 3×10^6 /ml and cultured in duplicate 1 ml aliquots for 2 hrs in Linbro 24 well flatbottom tissue culture plates. Adherent monolayers were then washed vigorously, and the medium in each well replaced with 1 ml of RPMI 1640 containing

5% FBS, 10 mM Hepes buffer, 20 ug/ml gentamicin, and 20 ug/ml LPS (lipopolysaccharide from S. typhimurium, Sigma Chemical Co., St. Louis, Missouri). After 20 hrs of incubation, culture supernatants were harvested, centrifuged at 900 x g for 15 minutes, and stored at 4^{o} C.

Cytocentrifuge slides of the PEC were taken prior to incubation of cultures, as described above.

Following harvest of culture supernatants, cell monolayers were stained and solubilized. Monolayers were covered in 0.5% crystal violet in 70% methanol for 1 minute, then rinsed in tap water. Culture plates were inverted and allowed to dry completely. Methyl Cellusolve (Fisher Scientific, San Francisco, CA) at 1.5 ml/well was added to each well and the plates were left to solubilize overnight. Serial dilutions of each solubilized monolayer were plated into a flatbottom 96 well microtiter plate and read on an ELISA reader at wavelength 540.

IL 1 Assays

The LBRM.TG6 bioassay was used to analyze all samples from the ex vivo model. The assay was performed as described by Larrick et al., (Larrick, Brindley & Doyle, 1985). This assay makes use of the LBRM.TG6

murine lymphoma cell line, which responds to IL 1 plus submitogenic levels of PHA by producing IL 2. LBRM.TG6 cells and IL 2 dependent CTLL cells are cultured together in serial dilutions of samples to be assayed for IL 1 content. The addition of hypoxanthine and azaserine to the cultures halts proliferation of LBRM.TG6. A subsequent pulse of tritiated thymidine (^{3}HTdR) allows measurement of the IL 2 dependent proliferation of CTLL, which serves as an indirect measure of the amount of IL 1 present.

The protocol is as follows. The medium used throughout is 10% IMDM supplemented with .02 mM 2-mercaptoethanol.

Supernatants were plated in triplicate serial dilutions in a flatbottom, 96 well microtiter plate at 100 ul/well. A standard of known high IL 1 content was also plated, as well as several wells containing medium with no IL 1 as a negative control. The standard used was purified human IL 1 (Collaborative Research Inc., Bedford, MA).

CTLL were washed 3 times before use to eliminate bound IL 2 and adjusted to 6×10^{5} cells/ml. LBRM.TG6 were adjusted to 4×10^{6} cells/ml. 25 ul aliquots of each cell type were added to each well. PHA (Burroughs-Wellcome, Greenville, North Carolina) at 5 ug/ml was

added in 50 ul aliquots to each well. The plates were incubated overnight. Hypoxanthine (Sigma Chemical Co., St. Louis, Missouri) at 22 x 10^{-4} M and azaserine (Sigma) at 220 ug/ml were then added to each well in 10 ul aliquots. After 4 hrs of incubation cultures were pulsed with 0.5 uCi ^{3}HTdR per well, and incubated another 4 hrs. Plates were then frozen, thawed and harvested using a Skatron semiautomatic cell harvester. Samples were counted on a beta counter using Instagel (Packard Instrument Co., Downers Grove, Illinois) as a fluor.

Data were analyzed using a program written by Bob Delongchamp of the Oregon State University Statistics Department. Values for each set of three replicates were averaged, and these values for each dilution and treatment group were averaged to give the mean counts per minute (cpm) for each treatment group and dilution. Results for each treatment group and dilution are also expressed as a percentage of the maximum response as determined by this calculation, where max response is the mean response produced by the highest concentration of IL 1 standard and background is the mean response produced by medium with no IL 1.

$$\frac{\text{mean response - background}}{\text{max response - background}} \times 100 = \% \text{ max response}$$

EL-4 Assay

Culture supernatants from the in vitro stimulation experiment were analyzed through the generosity of Dr. Paul Conlon and Sue Tyler of Immunex Corp., Seattle, Washington. They used a bioassay originally described by Simon et al., (Simon, Laydon & Lee, 1985). This assay uses the EL-4 mouse lymphoma, which produces IL 2 when stimulated simultaneously with IL 1 and the calcium ionophore A23187. The assay is analogous to the LBRM.TG6 assay, but involves a two-step process where the first step is IL 1 dependent IL 2 production and the second step is the assay of the resultant culture supernatants for IL 2 using the IL 2 dependent CTLL cell line. Data were analyzed as described for the LBRM.TG6 assay.

IL 2 Production

Spleen cell preparation

For all experiments on IL 2 production, single cell suspensions of splenocytes were prepared by teasing the spleens with forceps in Hanks Balanced Salt Solution supplemented with 5% FBS, 10 mM Hepes buffer, and 20 ug/ml gentamicin (5% HBSS). Dead cells and erythrocytes were lysed by a 10 second treatment with distilled water, and cellular debris was allowed to settle for 10

minutes. Spleen cell suspensions were kept cold throughout preparation. Cells were cultured in 10% RPMI.

Development of an IL 2 Production Model

Since no IL 2 was detectable in the PEC culture supernatants used for the ex vivo IL 1 production model, the following alternative approaches were used to measure IL 2 production by lymphocytes from P815 challenged animals. IL 2 has a very short halflife in vivo, due only in part to its adsorption by T cells (Muhlradt & Opitz, 1985). Various approaches were tried in the hope that lymphocytes activated in vivo would produce IL 2 in vitro which would accumulate to sufficient levels to be detected by bioassay.

Culture of splenocytes without additional in vitro stimulus

P815 injected animals were sacrificed on days 4 and 7 of the immune response. Spleens were removed and single cell suspensions of splenocytes were prepared as described.

Spleen cells were cultured in 10% RPMI in 2.5 ml aliquots in Linbro 24 well flatbottom tissue culture plates. Duplicate wells for each animal were set up at

5×10^6 cells/ml and 1×10^6 cells/ml. Cultures were incubated 24 hrs. Supernatants were then collected and stored at 4^{o} C until they could be assayed for IL 2.

No IL 2 was detected in this system, although the IL 2 assay was working well as demonstrated by the response to IL 2 standard.

Culture of splenocytes with mitomycin-inactivated P815

In this approach, spleen cells from P815 challenged mice were also stimulated *in vitro* with metabolically inactivated P815 cells. It was hoped that the continued stimulation *in vitro* would cause increased production of IL 2 so that it would reach detectable levels in culture supernatants.

Mice were sacrificed on day 3 of the immune response, and single cell suspensions of spleen cells prepared as described. Cultures were set up at four responder:stimulator ratios (100:1, 10:1, 1:1, 1:4) and supernatants were harvested at 12, 22, 37, 48, 72 and 96 hours of culture. No IL 2 was detected in any of these situations.

In vitro stimulation with Con A

Alloimmunized HxCB-exposed and control mice, 5 per

treatment group, were sacrificed on days 3, 5, and 7 of the immune response. Body and spleen weights were recorded for each mouse. Single cell suspensions of splenocytes were prepared as described.

Spleen cells at 2 x 10^6 cells/ml were cultured in 10% RPMI in horizontal 75 cm^2 culture flasks with 2.5 ug/ml Con A (Calbiochem, San Diego, CA). After 21 hr supernatants were harvested and cells removed by centrifugation. Supernatants were stored at 4 ^oC.

In this system high levels of IL 2 were detected in culture supernatants.

IL 2 Assay

IL 2 content was assayed by measuring the IL 2-dependent proliferation of the murine T cytotoxic cell line, CTLL.

The medium used was 10% RPMI. Serial dilutions of supernatants to be tested were plated in triplicate in a 96 well flatbottom microtiter plate at 100 ul/well. A purified human IL 2 standard of known high IL 2 content was also plated (Electronucleonics Inc., Silver Spring, Maryland). Several wells with medium only were plated as a negative control.

CTLL were washed 3 times before use to eliminate any bound IL 2. Cells were adjusted to 1 x 10^5 /ml and

aliquots of 100 ul added to each well. Cultures were incubated 20 hrs, then pulsed with 0.5 uCi ^{3}HTdR per well. After 4 hrs of incubation, cultures were frozen, thawed and harvested, and the data analyzed, as described under IL 1 assays.

IL 2 Responsiveness Model

The goal of these experiments was to test whether *in vivo* exposure to HxCB affects the ability of lymphocytes to proliferate in response to IL 2. The model is based on the work of Spitz et al., (Spitz, Gearing, Callus, Spitz & Thorpe, 1985) in which spleen cells and lymph node cells of animals challenged with sheep red blood cells (SRBC) were shown to proliferate in response to exogenous IL 2 *in vitro*.

Single cell suspensions of spleen and lumbar lymph node cells were prepared by teasing with forceps in 5% HBSS. Cells were pelleted, resuspended in 3 ml, and cellular debris allowed to settle for 10 min. Cells were washed 2 times to eliminate any bound IL 2, resuspended in 2 ml 10% RPMI, and counted on the Coulter counter. Cell concentrations were adjusted to 6×10^{6} spleen cells/ml and 3×10^{6} lymph node cells/ml. Lymph nodes from 3 to 4 animals were pooled to obtain enough

cells for one sample. Spleen cells from each animal were treated as separate samples.

Aliquots (50 ul) of the cell suspensions were added to triplicate wells in a 96 well flatbottom microtiter plate containing 50 ul of either medium alone or a 1/30 dilution of purified human IL 2. Plates were incubated 18 hrs, pulsed with 0.5 uCi ^{3}HTdR per well, incubated another 6 hrs, then frozen, thawed and harvested as described under IL 1 assays.

RESULTS

Preliminary

The initial goal of these experiments was to determine whether *in vivo* exposure to HxCB affects the ability of monocytes to produce IL 1 or of lymphocytes to produce IL 2 and respond to IL 2 by proliferating. We attempted to develop a system for direct measurement of interleukin levels at the site of the immune response (the peritoneal cavity) in P815-injected mice. To test this possibility, C57Bl/6 mice were injected with P815 or vehicle and sacrificed at 6, 12, and 24 hours after injection. Peritoneal exudates were harvested using 5 ml culture medium supplemented with heparin, and subjected to the following treatments: 1) exudate was centrifuged and the supernatant assayed for interleukins without further treatment, 2) exudate, still containing peritoneal exudate cells (PEC), was incubated for 24 hours in 1.5 ml aliquots, after which the culture supernatant was collected and assayed for interleukins, 3) exudate was centrifuged, the supernatant discarded and the PEC resuspended in heparin-free medium, then incubated for 24 hours and the culture supernatant assayed for interleukins.

In this preliminary study, IL 1 was not detected in

peritoneal exudates straight out of the animals (treatment 1), but was detected in culture supernatants from treatments 2 and 3. IL 1 activity was higher in supernatants of cells which had not been resuspended, indicating that the heparin in the medium did not inhibit IL 1 production. No IL 2 was detected in any of the three treatment situations.

Subsequent experiments on interleukin production followed the basic model described as treatment 2 above, and peritoneal exudates from HxCB treated and control animals at 6, 12, 24, 54, 72 and 96 hours of the immune response were collected, incubated and tested for interleukin content. IL 1 was detected at all of these timepoints. IL 2 was not detected at any timepoint.

Following these experiments, separate approaches were developed to address the questions of IL 2 production and response to IL 2. An additional experiment testing the ability of peritoneal adherent cells of HxCB exposed and control animals to produce IL 1 in response to an *in vitro* stimulus was performed to supplement the *ex vivo* model described above.

IL 1 Production

Ex vivo model

Peritoneal exudates of alloimmunized, HxCB treated

and control mice as well as nonimmune controls were incubated 24 hrs and the culture supernatants assayed for IL 1 content. Cytospin slides of the PEC from each animal were made and stained to allow the differentiation of PEC into macrophages, P815 cells, lymphocytes and granulocytes.

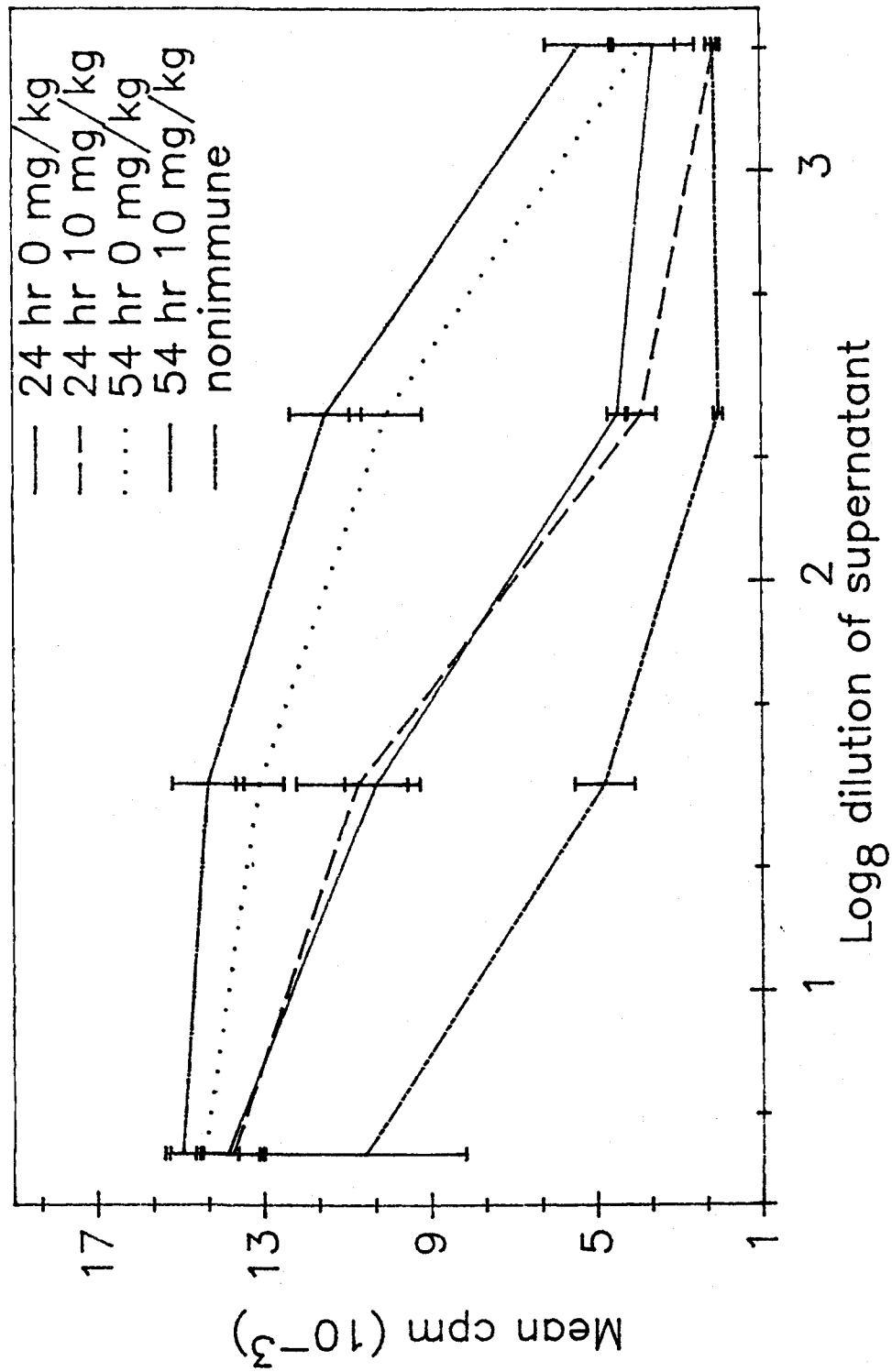
The results of a typical IL 1 assay are shown in Fig. 1. At each timepoint (6, 12, 24, 54, 72 and 96 hours post-P815) dose-response curves in the IL 1 assay were parallel. There were no significant differences in ^{3}HTdR incorporation between HxCB treated and control groups at any timepoint. Nonimmune PEC cultures consistently produced low responses in the IL 1 assay.

Because there is variation in the extent of ^{3}HTdR incorporation each time an interleukin assay is run, a direct comparison of the cpm obtained in separate IL 1 assays could be misleading. The IL 1 data for this study were obtained in three separate assays. In order to compare IL 1 activity at the different timepoints, results were expressed as a percentage of the maximum response to the IL 1 standard in each assay (see Methods).

Fig. 2 shows the kinetics of IL 1 production in this model expressed as percent maximum response at a 1/32 dilution of the peritoneal exudate culture

Fig. 1. IL 1 assay results at 24 and 54 hrs of the immune response to P815. Maximum response to the IL 1 standard was 16989 cpm. Results are typical in that dose-response curves for the two alloimmune treatment groups (0 mg/kg and 10 mg/kg HxCB) are parallel with no significant differences in 3HTdR incorporation, while the level of IL 1 production in nonimmune animals is comparatively low. Error bars represent standard error.

Figure 1.


24 hr 0 mg/kg
24 hr 10 mg/kg
54 hr 0 mg/kg
54 hr 10 mg/kg
nonimmune
Mean cpm (10^{-3})
17
13
9
5
1
1
2
3
Log_8 dilution of supernatant

supernatants from each timepoint. There are no significant differences between HxCB exposed and control groups. The response for the nonimmune control group is consistently low. This indicates that while there may be an ongoing production of IL 1 by peritoneal macrophages in naive animals, the increased levels of response seen in P815 injected animals at varying times after P815 exposure are due to the immune response against P815. IL 1 production rises significantly after 12 hrs, peaks at 54 hrs, and declines over the following timepoints.

To determine whether IL 1 detected in this system was produced by the P815 cells themselves, peritoneal exudate from a DBA mouse carrying the tumor was cultured and the supernatant assayed for IL 1. The results of this experiment are presented in Table 1. PEC were cultured at two cell concentrations comparable to those found in exudates of C57Bl/6 subjects at 24 and 54 hours of the immune response. Separate cultures were incubated for 24 or 48 hrs before harvest of supernatants. The percent max response produced by these samples is comparable to that produced by samples from nonimmune C57Bl/6 mice. This low level of IL 1 activity is probably due to normal baseline IL 1 production by resident peritoneal macrophages of the DBA mouse.

Fig. 2. Kinetics of IL 1 production in the ex vivo model. To allow comparison of IL 1 levels measured in separate assays, results are expressed as the percent maximum response induced by a 1/32 dilution of sample at each timepoint. Error bars represent standard error.

Figure 2.

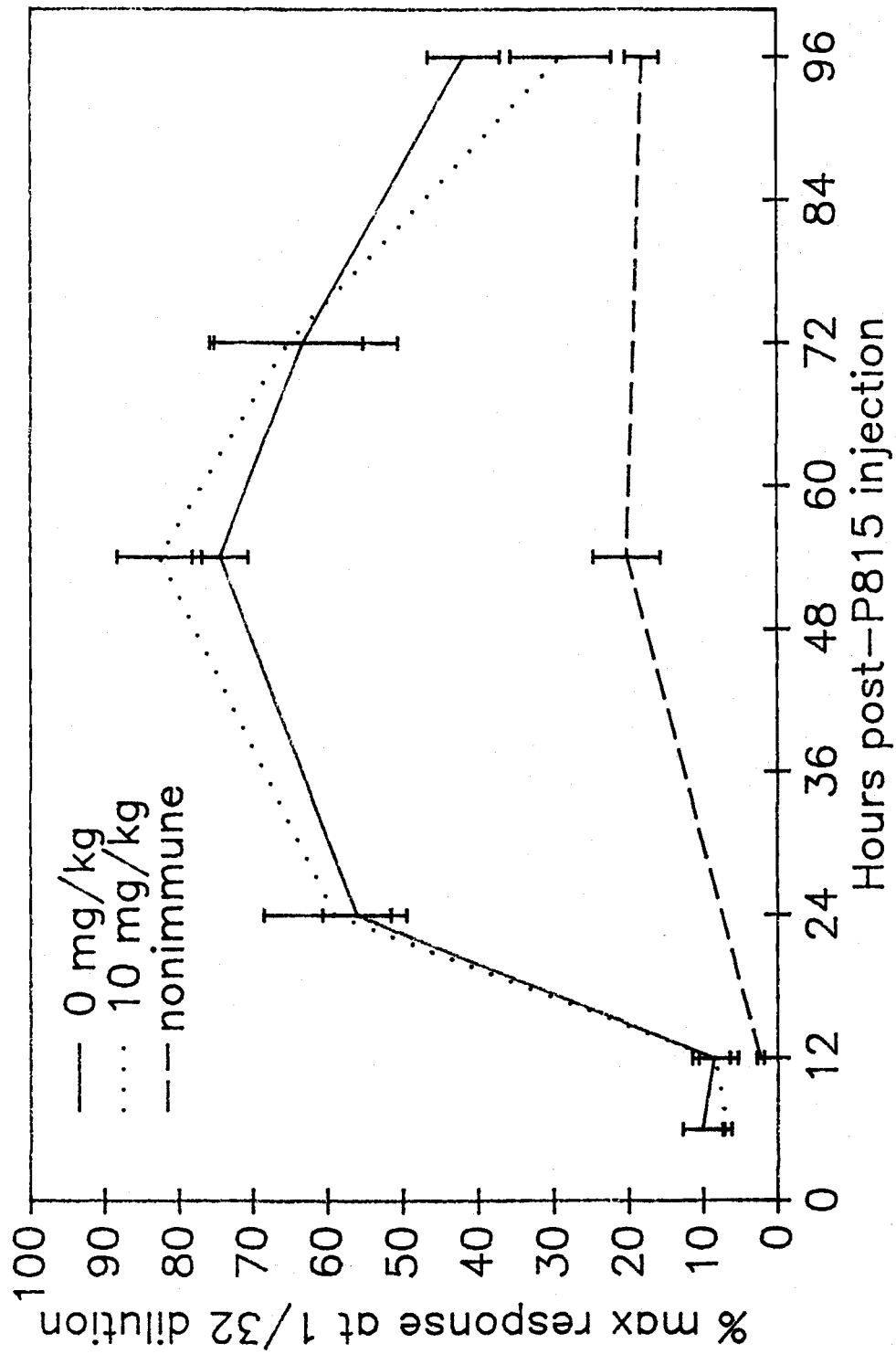

% max response at 1/32 dilution
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
84
96
Hours post-P815 injection
0 mg/kg
10 mg/kg
nonimmune

Table 1. Production of IL 1 in P815 cell cultures. Percent maximum response at a 1/32 dilution of each sample is shown.

Cell conc.	Hours cultured	Mean cpm	% Max resp
$1x10^6$	24	2100	12.0
$2x10^5$	24	2050	11.0
$1x10^6$	48	1840	6.4
$2x10^5$	48	2250	15.4

Results of the cell differential counts at each timepoint of the ex vivo study are presented in Fig. 3. There was a gradual increase in the numbers of both macrophages and P815 cells recovered over time. The numbers of lymphocytes and granulocytes recovered remained low at the latest timepoint of 96 hrs post-P815. There were no significant differences between HxCB treated and control groups in the numbers of any cell type recovered.

Peak IL 1 production was seen at 54 hrs, while the number of macrophages began to increase more rapidly after 72 hrs. Previous studies in this laboratory (Kerkvliet & Baecher-Steppan, in press) have shown that the major influx of macrophages to the peritoneal cavity takes place on day 8-9 of the response to P815. The data from the present study suggest that the peak of IL 1 production at 54 hrs is due to production by resident peritoneal macrophages involved in activation of T cells. The large influx of macrophages to the peritoneal cavity in the later stages of the immune response would likely consist of recruited macrophages, activated to become cytotoxic by lymphokines released by activated T cells (Roitt, Brostoff & Male, 1985).

Fig. 3. Number of macrophages, P815 cells, lymphocytes and granulocytes recovered at each timepoint examined in the ex vivo IL 1 production model. Cell concentrations were determined by Coulter counter at the initiation of cultures. At the same time cytospin slides were made and used to ascertain the percentage of each cell type in the sample. Uniform volumes of peritoneal exudate were cultured to allow calculation of the number of each cell type per sample. Error bars represent standard error.

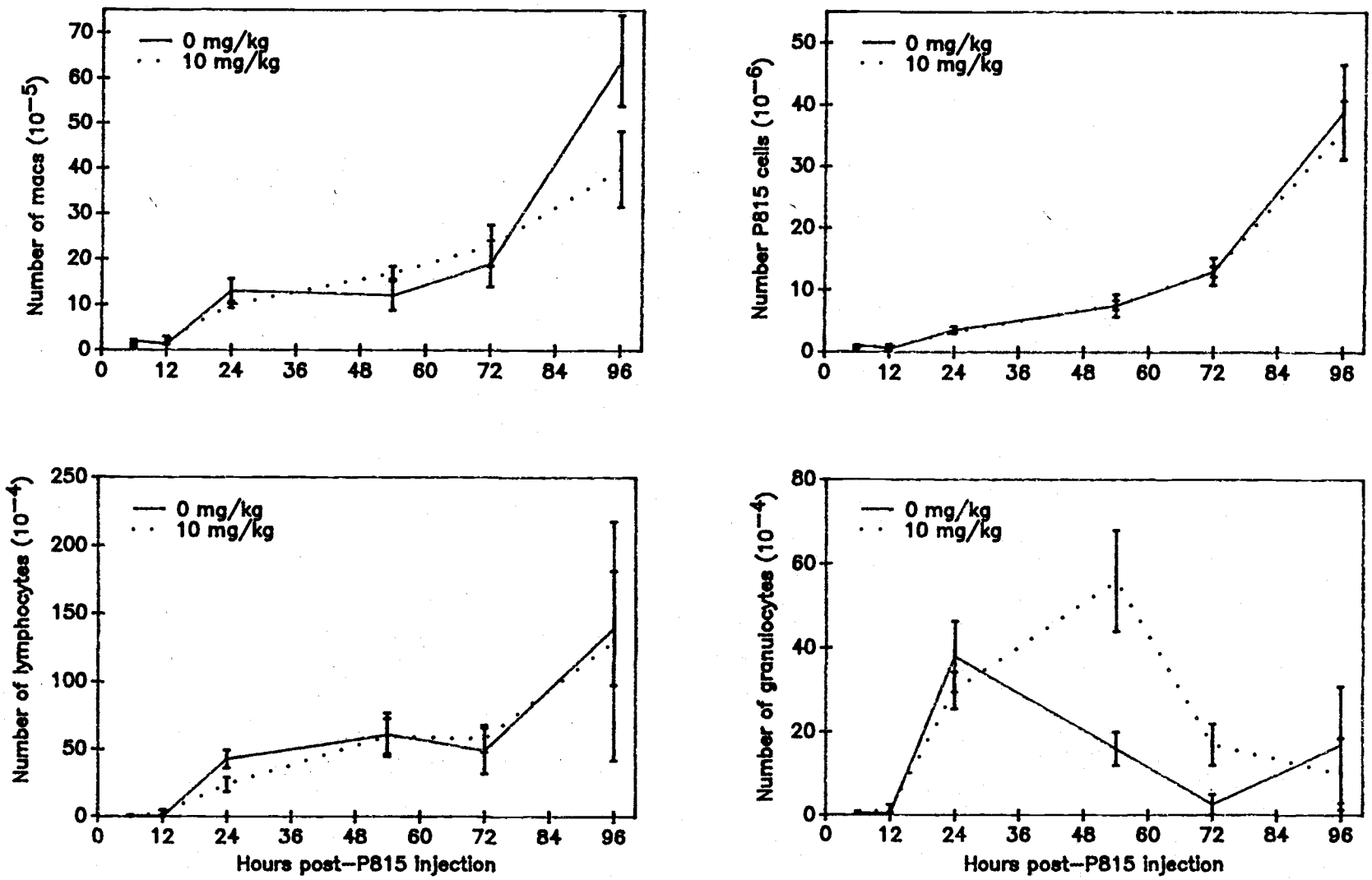

Number of macs (10^{-5})
0 mg/kg
10 mg/kg
Number P815 cells (10^{-6})
Number of lymphocytes (10^{-4})
Number of granulocytes (10^{-4})
Hours post-P815 injection
Hours post-P815 injection

Figure 3.

IL 1 in vitro model

To supplement the results obtained in the ex vivo IL 1 production model, thioglycollate-elicited peritoneal macrophages from HxCB treated and control mice were tested for their ability to produce IL 1 in response to in vitro stimulation with LPS.

Results of the IL 1 assay of these samples conducted at Immunex Corp. are shown in Fig. 4. As in the ex vivo model, dose-response curves for the two groups were parallel, with no significant differences between groups.

Information on the cells cultured from each treatment group is shown in Tables 2 and 3. Solubilization of adherent cell monolayers indicated no significant differences in the number of adherent cells per culture. Likewise, results of cell differential counts showed no significant differences in the percentages of each cell type recovered.

IL 2 Production

Spleen cells of alloimmune HxCB exposed and control mice were harvested on days 3, 5, and 7 of the immune response and stimulated in vitro with Con A for 21 hrs. The culture supernatants were then harvested and assayed for IL 2.

Fig. 4. IL 1 production by adherent thioglycollate-elicited PEC cultures stimulated *in vitro* with LPS. Assay was performed by Dr. Paul Conlon's laboratory at Immunex Corp. Error bars represent standard error.

Figure 4.

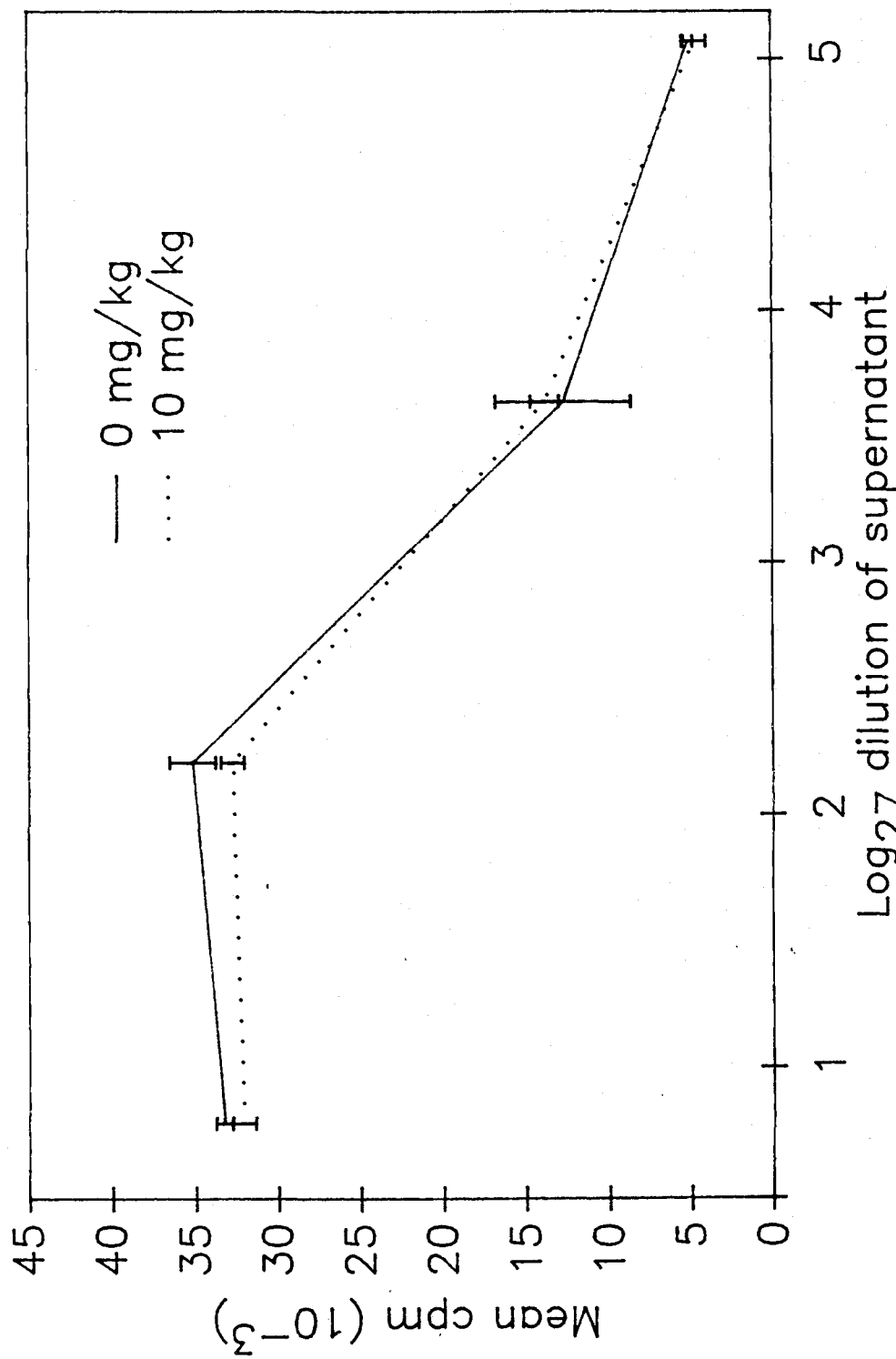

— 0 mg/kg
····· 10 mg/kg
Mean cpm (10^{-3})
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
Log_{27} dilution of supernatant

Table 2. Subpopulations of PEC recovered in the *in vitro* IL 1 production model.

Trtmt	% Macs	sd	% Gran	sd	% Lympho	sd
0 mg/kg	78.9	5.8	14.8	4.7	6.25	1.4
10 mg/kg	83.9	5.5	9.8	3.6	6.25	2.4

Table 3. Mean optical density of solubilized adherent PEC monolayers from the *in vitro* IL 1 production model.

Treatment	Mean OD	sd
0 mg/kg	.623	.096
10 mg/kg	.578	.150

Results of a typical IL 2 assay are shown in Fig. 5. Dose-response curves for the two treatment groups were parallel, and there were no significant differences in ^{3}HTdR incorporation between groups. This was the case for all three timepoints.

IL 2 production at the three timepoints is compared in Fig. 6, in terms of percent maximum response at a 1/8 dilution (see Methods, IL 1 assays). Levels of production were highest at day 3, with a gradual decrease over days 5 and 7.

Spleen weight and body weight data for these experiments are presented in Table 4. There were no significant differences between groups in spleen cell recovery or in spleen weight when expressed as a percent of body weight.

IL 2 Response

Spleen and lumbar lymph node cells (the lumbar nodes drain the peritoneal cavity) were taken from nonimmune animals as well as alloimmune HxCB exposed and control mice and cultured for 24 hrs, including a 6 hr pulse with ^{3}HTdR. Each sample was cultured with and without exogenous IL 2. The proliferative responses of spleen cells and lymph node cells followed similar patterns.

Fig. 5. IL 2 assay of Con A stimulated splenocyte culture supernatants. Splenocytes were collected on day 3 after P815 injection. Results are typical, with parallel dose-response curves for the two treatment groups and no significant differences in 3HTdR incorporation. Error bars represent standard error.

Figure 5.

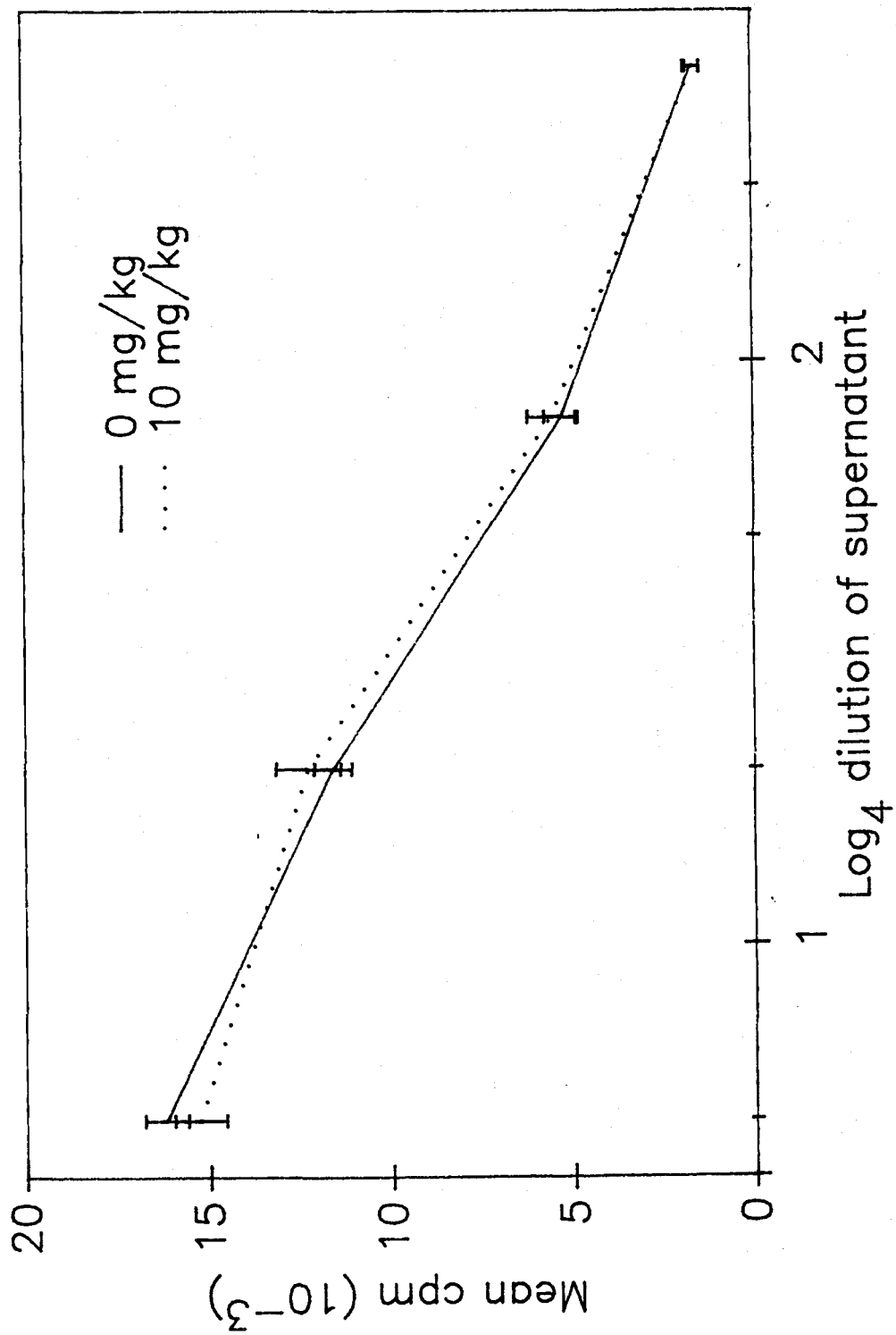

— 0 mg/kg
···· 10 mg/kg
Mean cpm (10^{-3})
20
15
10
5
0
1
2
Log_4 dilution of supernatant

Fig. 6. Kinetics of IL 2 production in the Con A stimulation model. To allow comparison of IL 2 levels measured in separate assays, results are expressed as the percent maximum response induced by a 1/8 dilution of sample at each timepoint. Error bars represent standard error.

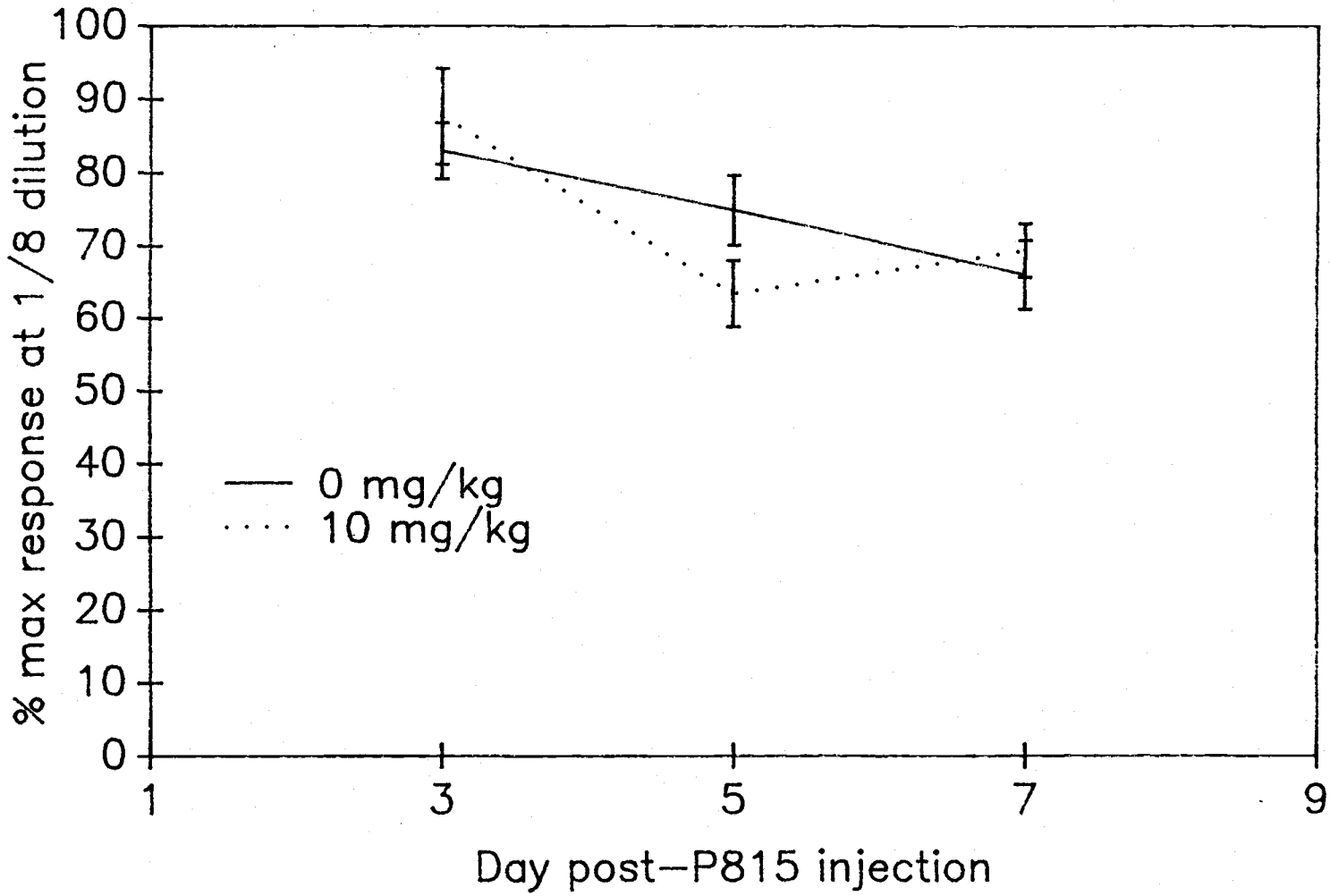

100
90
80
70
60
50
40
30
20
10
0
% max response at 1/8 dilution
0 mg/kg
10 mg/kg
1
3
5
7
9
Day post-P815 injection

Figure 6.

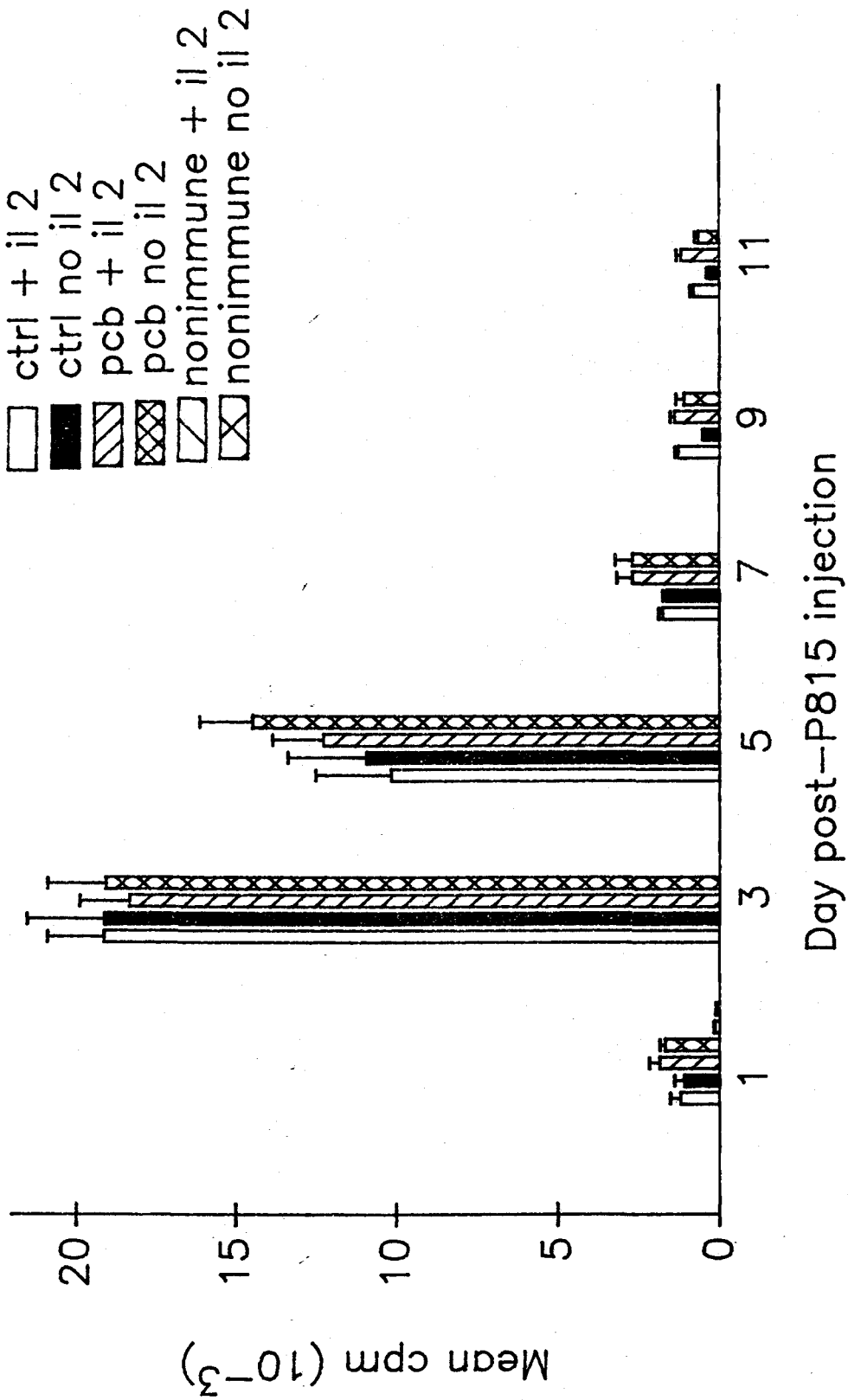
Table 4. Spleen and body weight data from mice used in the Con A stimulated IL 2 production model. Error is standard error.

Day	Trtmt	BW(g)	SW(mg)	SW/BW(mg/g)	SCx10^7
3	0	25.72±.39	87±4	3.38±.12	7.16±.45
3	10	25.75±.67	89±4	3.47±.11	6.45±.23
5	0	26.23±.74	161±6	6.13±.15	6.9±.61
5	10	23.49±.34	141±5	5.99±.29	6.6±.26
7	0	23.97±.96	116±9	4.9±.31	6.5±.27
7	10	21.78±.62	104±5	4.8±.44	5.8±.55

Spleen cell results are shown in Fig. 7. No response to exogenous IL 2 was seen until days 9 and 11. For both HxCB and control groups the proliferative response was unaffected by exogenous IL 2 on days 1-7. Nevertheless there was clearly a strong proliferative response in both alloimmune treatment groups which occurred with or without the addition of IL 2 to the cultures, and which did not occur in nonimmune animals. This response occurred as early as day 1, peaked on day 3 and declined gradually over the succeeding timepoints. This "spontaneous proliferation" appears to be due to in vivo activation of lymphocytes against alloantigen. The pattern of response shown here is supported by preliminary experiments done for days 3, 5, and 7. In these experiments the spontaneous proliferation was also highest on day 3, decreasing on days 5 and 7, while the baseline response of nonimmune animals was similar. There was no response to exogenous IL 2 in a series of preliminary experiments in which several different concentrations of IL 2 were tested. The concentration of IL 2 used in the experiments presented here was a 1/30 dilution of purified human IL 2, the same concentration which was used to maintain the IL 2-dependent murine T cytotoxic cell line, CTLL.

Fig. 7. Proliferation of splenocytes from alloimmune and nonimmune animals cultured with and without exogenous IL 2. Error bars represent standard error.

Figure 7.


ctrl + il 2
ctrl no il 2
pcb + il 2
pcb no il 2
nonimmune + il 2
nonimmune no il 2
Mean cpm (10^{-3})
0
5
10
15
20
1
3
5
7
9
11
Day post-P815 injection

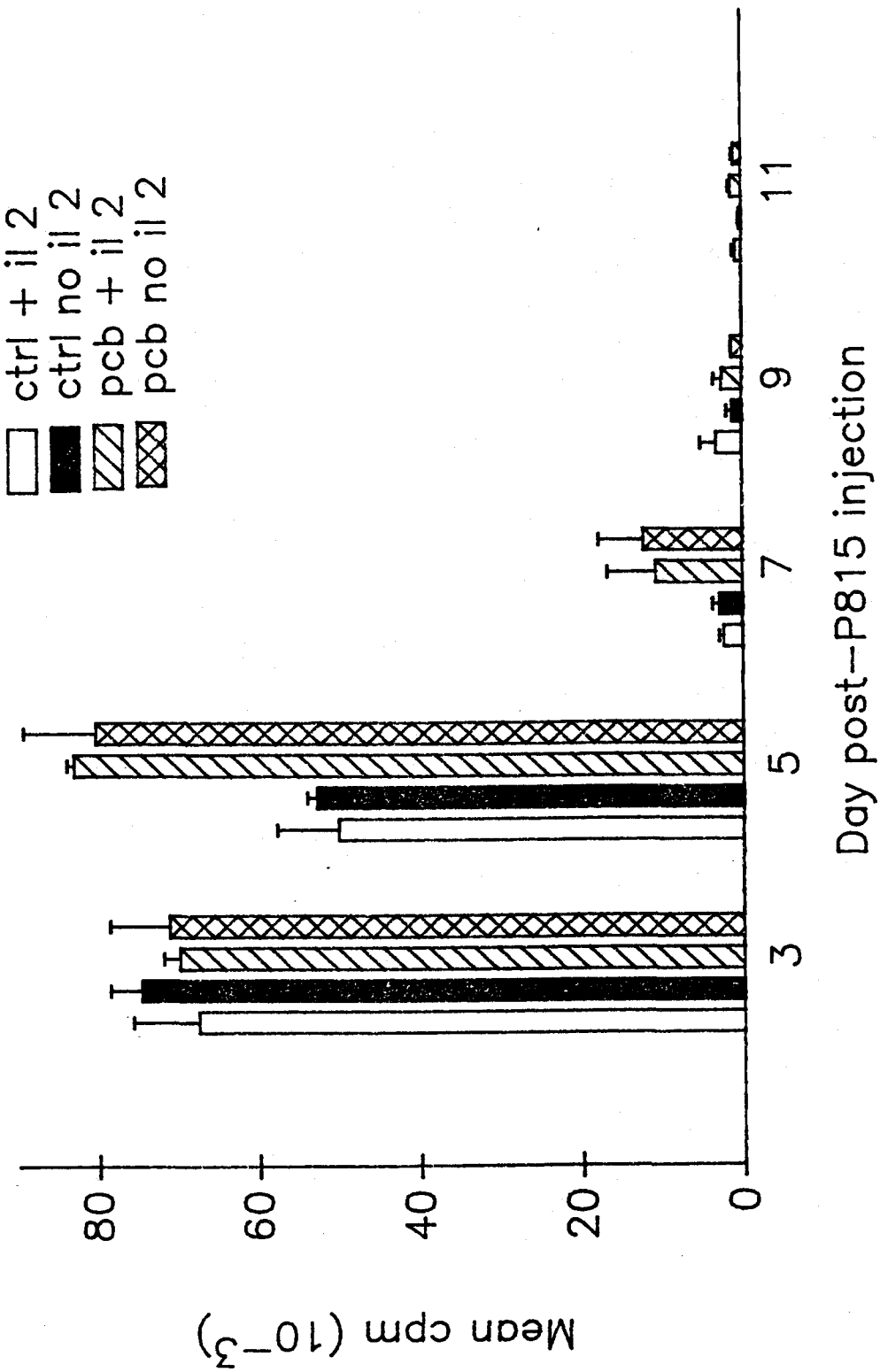
Days 9 and 11, when the spontaneous proliferation response was at low levels, were the only timepoints at which there was a significant response to exogenous IL 2. For the control group, the increase in ^{3}HTdR incorporation was significant on both days at a level of 0.001. The same trend appeared on these two days in the HxCB group, but the response to exogenous IL 2 was not significant on either day.

The proliferative response of lymph node cells is shown in Fig. 8. The same trends seen in the spleen cell response appeared with lymph node cells, although ^{3}HTdR incorporation for lymph node cells was up to 4 times greater than that of spleen cells at each timepoint. The results shown represent the mean values from triplicate cultures of 2-3 pools of lymph node cells for each treatment group and timepoint.

As with spleen cells, the peak of spontaneous proliferation of lymph node cells was on day 3, with a gradual decrease over the succeeding days. The spontaneous proliferation of the HxCB treated group was significantly higher than that of the control group on days 5 and 7. No significant response to exogenous IL 2 was seen, although the trend toward development of a response to IL 2 in the control group on days 9 and 11 was observed.

Fig. 8. Proliferation of lumbar lymph node cells from alloimmune animals cultured with and without exogenous IL 2. Lymph nodes of 3-4 animals were pooled. There were 2-3 pools per treatment group and timepoint. Error bars represent standard deviation.

Figure 8.


ctrl + il 2
ctrl no il 2
pcb + il 2
pcb no il 2
Mean cpm (10^{-3})
0
20
40
60
80
3
5
7
9
11
Day post-P815 injection

Preliminary experiments with lymph node cells, which used only one cell pool per treatment group, confirmed this overall pattern. A significantly higher spontaneous response of the HxCB treated group on day 7 was verified in two repetitions, although a higher spontaneous response of this group on day 5 was not supported.

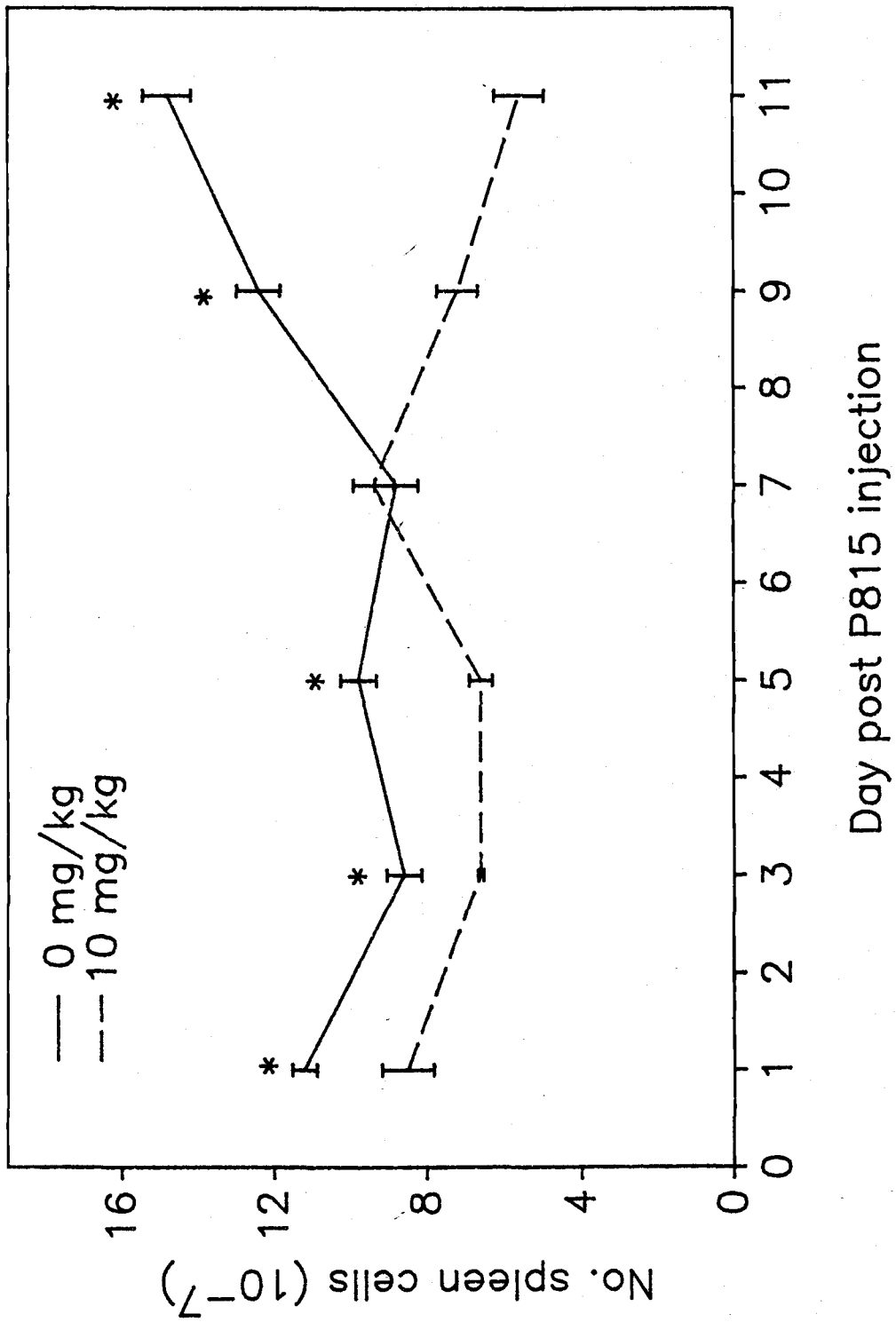
Spleen and body weight data for the series of experiments presented here are given in Table 5. The graph of spleen cell recovery over time (Fig. 9) reflects a phenomenon established in earlier studies in this laboratory. The spleen weights and cellularity of the HxCB-exposed animals remained constant, while both values increased in the control group after day 7.

Table 5. Spleen and body weight data for mice used in the IL 2 response model. Error is standard deviation.

Trtmt	Day	BW(g)	SW(mg)	SW/BW(mg/g)	$SCx10^7$
Nonim	--	23.49±2.8	78±13	3.2±0.2	10.8±2.2
0	1	23.46±1.4	78±8.8	3.3±0.2	11.2±0.8
10	1	21.25±0.6	68±10	3.2±0.5	8.5±1.5
0	3	25.20±1.9	77±11	3.0±0.2	8.6±1.4
10	3	23.95±1.2	60±7	2.5±0.3	6.6±0.9
0	5	26.30±1.6	104±16	3.9±0.7	9.8±1.5
10	5	24.70±1.1	79±9	3.2±0.3	6.6±0.9
0	7	31.77±3.1	118±14	3.6±0.2	8.8±1.5
10	7	27.51±2.0	85±13	3.0±0.5	9.4±1.4
0	9	32.66±3.7	206±25	6.3±1.0	12.4±1.4
10	9	23.70±0.8	123±28	5.1±1.0	7.2±1.4
0	11	29.87±2.3	158±20	5.3±0.5	14.8±1.7
10	11	23.67±1.8	73±15	2.9±0.5	5.6±1.8

Fig. 9. Spleen cell recovery during the IL 2 response study. Asterisks indicate significantly higher recovery in the control group. Error bars represent standard error.

Figure 9.


No. spleen cells (10^{-7})
16
12
8
4
0
0 1 2 3 4 5 6 7 8 9 10 11
Day post P815 injection
— 0 mg/kg
– – 10 mg/kg
*

DISCUSSION

Ah receptor interacting PCB isomers are immunosuppressive in sensitive strains of mice such as C57Bl/6. HxCB has been used in this laboratory as a prototype immunosuppressive isomer. Previous studies have provided evidence that HxCB inhibits the *in vivo* generation of cytotoxic activity against alloantigen by altering event(s) early in the immune response. The production of and response to interleukins 1 and 2 are crucial early events in the activation of CTL. In the present study, models were developed to evaluate IL 1 production, IL 2 production, and the proliferative response to IL 2 in HxCB treated and control C57Bl/6 mice. The model system used throughout was the rejection of the allogeneic P815 mastocytoma. In the case of IL 1 production, the ability of PEC to respond to *in vitro* stimulation with LPS was also examined. Likewise, IL 2 production in response to Con A was tested.

HxCB did not significantly alter IL 1 production by PEC harvested at varying timepoints of the immune response to P815, nor were subpopulations of recovered PEC significantly altered. In comparison to baseline levels of IL 1 production by PEC from naive animals, IL 1 production in response to P815 increased rapidly after

12 hrs, peaked at 54 hrs and declined until 96 hrs, the latest timepoint tested. Since this wave of IL 1 production precedes by several days the major influx of P815-activated macrophages into the peritoneal cavity (Kerkvliet & Baecher-Steppan, in press), it appears to be due to IL 1 secretion by resident peritoneal macrophages.

Thioglycollate has been frequently used as an inflammatory agent to increase the yield of peritoneal macrophages. The macrophages elicited by thioglycollate differ in several characteristics from naive resident peritoneal macrophages (Bloom & David, 1976). Elicited macrophages have an increased content of lysosomal granules, altered levels of activity for several enzymes, and an increased pinocytotic rate. While such cells have been used successfully in a number of tests of macrophage function, they cannot be strictly equated with naive cells.

Results using thioglycollate-elicited macrophages show no significant alteration of IL 1 production or of PEC subpopulations by HxCB exposure. Thus the results of the two IL 1 production models are in agreement, indicating that HxCB does not exert its suppressive effects through an inhibition of IL 1 production.

IL 2 is an extremely short-lived mediator under

physiological conditions (Forni et al., 1986; Muhlradt & Opitz, 1982). The development of a model for the evaluation of IL 2 production in response to antigen was complicated by the ephemeral nature of IL 2.

Efforts to produce measurable IL 2 in culture supernatants of spleen cells from alloimmune animals cultured for various periods of time with or without mitomycin C inactivated P815 cells were unsuccessful. The failure of continued in vitro stimulation with alloantigen to produce IL 2 was unexpected, since both primary and secondary mixed lymphocyte cultures (MLC) have been used to produce IL 2 (Farrar, Johnson & Farrar, 1981; Bocchieri, Knittweis & Seaton, 1981). However, the splenocyte-P815 cultures used in this study differed from a MLC in that the stimulator used was an Ia negative mastocytoma, rather than an Ia positive lymphocyte.

Similar models in the literature report varying success with the use of different antigens and sources of lymphoid cells to demonstrate IL 2 production. Spitz et al., (1985) demonstrated in vitro production of IL 2 by splenocytes, which peaked on day 3 following an intrasplenic injection of SRBC. In the same study, draining lymph node cells of mice given a footpad injection of SRBC spontaneously produced IL 2 in

culture. Spleen and other lymphoid cells of mice undergoing an infection of Nematospiroides dubius, a parasitic worm, produced IL 2 when cultured with somatic antigens of N. dubius but not of other helminths (61). Such IL 2 secreting cells appeared in the lymph nodes by day 5 of a primary infection. These studies demonstrate that the idea of testing antigen-specific IL 2 production by culturing lymphocytes activated in vivo can be workable in some circumstances. A future possibility for the P815 model might be testing in vitro IL 2 production of lymphocytes from nodes draining the peritoneal cavity.

Results of experiments in which spleen cells of alloimmune mice were stimulated in vitro with Con A showed no significant differences in IL 2 production between HxCB exposed and control groups. While high levels of IL 2 were found whether mice were sacrificed on day 3, 5, or 7 of the immune response, the highest levels occurred on day 3, declining on days 5 and 7.

Because Con A is a polyclonal stimulator of T cells, these data do not reflect antigen-specific IL 2 production. High levels of IL 2 due to polyclonal production could conceivably mask suppression of antigen-specific IL 2 production. Mitogenic stimulation has however been frequently used to assess IL 2

production capabilities (House, Lauer, Murray & Dean, 1987; Exon, Talcott & Koller, 1985; Bocchieri, Knittweis & Seaton, 1984). 7,12-dimethylbenzanthracene suppressed the *in vitro* generation of cytotoxic activity against alloantigen and significantly suppressed IL 2 production (House, Lauer, Murray & Dean, 1987). In that study splenocytes of toxicant exposed and control mice were cultured 48 hrs with Con A before harvest and IL 2 assay of the culture supernatants. A study of the effects of the commercial PCB mixture Aroclor 1254 on IL 2 production produced conflicting results (Exon, Talcott & Koller, 1985). *In vitro* exposure to Aroclor decreased Con A stimulated IL 2 production while *in vivo* exposure increased it. We have been unable to find any previous studies of the effect of purified PCB isomers on IL 2 production.

The problems inherent in the use of mitogenic stimulation for an IL 2 production model, as well as the conflicting results of previous studies, make it difficult to draw a definite conclusion as to whether IL 2 production is affected by HxCB exposure. However, data presented here which show no significant inhibition by HxCB of the proliferative response of spleen and lymph node cells activated *in vivo* against P815 would support the finding of no effect on IL 2 production.

The IL 2 response model used in these studies was based on the work of Spitz et al., (Spitz, Gearing, Callus, Spitz & Thorpe, 1985). These investigators administered footpad or intrasplenic injections of SRBC and subsequently tested the proliferative response of spleen and lymph node cells to exogenous IL 2 in culture. Their results clearly indicate that lymphocytes activated by SRBC in vivo have an increased proliferative response in the presence of exogenous IL 2 which varies according to the timepoint in the immune response. Levels of spontaneous proliferation were consistently low.

In our model HxCB treated and control mice were injected i.p. with P815 cells, and the spleen and lumbar lymph node cells were harvested at six timepoints during the immune response. Cells were cultured with and without exogenous IL 2. In contrast to the results obtained by Spitz et al., (1985) levels of spontaneous proliferation varied widely according to the day of the immune response. Peak proliferation occurred on day 3 for both spleen and lymph node cells, declining thereafter. Exogenous IL 2 had no significant effect on ^{3}HTdR incorporation except on days 9 and 11 in the control spleen cell cultures. HxCB exposure did not inhibit the proliferative response overall. The HxCB

treated group appeared to be a step behind the control group at several timepoints for both spleen and lymph node cells. A slight delay in the response of HxCB treated animals could explain why the spontaneous proliferation of this group was higher than that of controls on day 7 in both cell types. On day 9 there was a significant response to exogenous IL 2 in the spleen cells of controls only, but the spontaneous response of the HxCB treated group was not significantly different from the IL 2 driven response of the controls. By day 11, although the response of the toxicant treated group to IL 2 was not significant, there was a trend toward a lowered spontaneous response.

The time course of spontaneous proliferation presented here indicates a response to alloantigen exposure in vivo. Spontaneous proliferation of lymphocytes from alloimmune animals was significantly higher than that of nonimmune animals at all timepoints, with peak proliferation observed on days 3 and 5. These high levels of spontaneous proliferation strongly suggest that the lymphocytes were activated by IL 2 generated in vivo, and were saturated with IL 2 so that added lymphokine did not affect the response. The effect of exogenous IL 2 observed late in the immune response (day 9-11) may indicate that in vivo IL 2

production had declined, so that the cell population is no longer saturated with IL 2.

The in vivo generation of a proliferative response requires the production of IL 1 and IL 2 as well as the ability to respond to both lymphokines (Henney et al., 1982). Thus the results of the IL 2 response experiments suggest that HxCB does not affect any of the three parameters of interest. However, results of parallel studies in this laboratory which used flow cytometry to examine surface marker expression of splenocytes seem to contradict this model (Kerkvliet & Brauner, unpublished). In control mice on day 10 of the immune response there was a significant increase in the percentage of both Thy 1.2+ and Ly 2+ cells, while the percentage of L3T4+ cells remained constant. Because the total number of spleen cells recovered from control mice significantly increased during the course of tumor rejection, the total number of cells expressing Thy 1.2, Ly 2, and L3T4 markers increased. No significant change in the percentage or total numbers of splenocytes expressing Thy 1.2, Ly 2, or L3T4 markers was seen in HxCB treated mice. These data suggest that HxCB prevented the normal activation and proliferation events that occur in response to allogeneic challenge. IL2R expression was examined on spleen cells harvested at

days 3, 5, and 7 of the immune response following coculture in vitro with Con A for 21 hrs. As compared to control mice, the percentage of L3T4+ cells expressing IL2R was significantly reduced in HxCB exposed mice on day 7 of the response, suggesting that the ability to respond to IL 2 may be negatively affected by HxCB.

It is difficult to reconcile the surface marker data with the spontaneous proliferation observed in the present study. It has been demonstrated repeatedly in this laboratory that an increase in spleen weight and cellularity is a normal consequence of P815 challenge, which fails to take place in HxCB exposed animals. Yet the in vitro proliferative response of lymphocytes from HxCB treated animals is seen to closely parallel that of controls. While the highest levels of spontaneous proliferation occurred on days 3 and 5 in both treatment groups, significant increases in spleen cellularity and in Thy 1.2+ and Ly 2+ cells occurred after day 7, and only in controls.

These changes in splenocyte subpopulations after day 7 may be due to recruitment of T cells from the thymus. P815 challenge alone causes a decrease in thymus weight (Kerkvliet & Baecher-Steppan, in press), which could be due to the redistribution of thymic lymphocytes.

Likewise the high levels of spontaneous spleen cell proliferation early in the response may respresent the growth of cells which are then exported from the spleen. Alloantigen challenge has been shown to elicit a humoral as well as a cell mediated immune response (Engers & Louis, 1979) so that proliferation of B cells may in part be responsible for the spontaneous response.

It might be instructive to examine surface marker expression of spleen cell populations both before and after spontaneous proliferation in vitro, to determine which subpopulations are increasing. Another possibility for future research might be the development of a system to measure in vivo lymphocyte proliferation, as by giving an injection of ^{3}HTdR and then measuring the extent of ^{3}HTdR incorporation in the spleen. Such experiments could establish whether the spontaneous proliferation seen here accurately reflects events in vivo.

CONCLUSIONS

Data obtained from both ex vivo and in vitro models indicated no effect of HxCB exposure on IL 1 production. The kinetics of IL 1 production following in vivo alloantigen challenge suggest a wave of production by resident peritoneal macrophages early in the immune response.

Development of a system to evaluate antigen-specific IL 2 production in HxCB exposed and control animals was complicated by the ephemeral nature of IL 2. The only model in which detectable levels of IL 2 were produced involved in vitro Con A stimulation of lymphocytes from alloimmune animals. Results of these experiments showed no effect of HxCB on IL 2 production.

The ability of lymphocytes to proliferate in response to antigen stimulation appeared to be unaffected by HxCB in the experimental model presented here. High levels of spontaneous proliferation of spleen and lymph node cells from alloimmune mice imply that saturating levels of IL 2 are generated in vivo.

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